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COMPENDIUM OF THE FY1988 & FY1989 RESEARCH REVIEWS FOR THE RESEARCH METHODS BRANCH

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Since 1983, the U.S. Army Biomedical Research and Development Laboratory (USABRDL) has conducted a research program focused on the development of new methods for assessing the carcinogenic hazard resulting from exposure to chemicals in the workplace or the environment. This research has been conducted both in-house and extramurally and has involved scientists from other Federal agencies, academic institutions, and the private sector. The need to enhance the integration of the research became increasingly evident as the program grew in the late 1980's. This growth came principally as a result of the U.S. Army Corps of Engineers sponsorship of research to develop environmental applications of new, in vivo cancer assessment models for use in the burgeoning Department of Defense environmental remediation program. The convening of an annual research review meeting was undertaken to facilitate the integration and technology transfer of this research program. This publication is a compendium of the first two such meetings.

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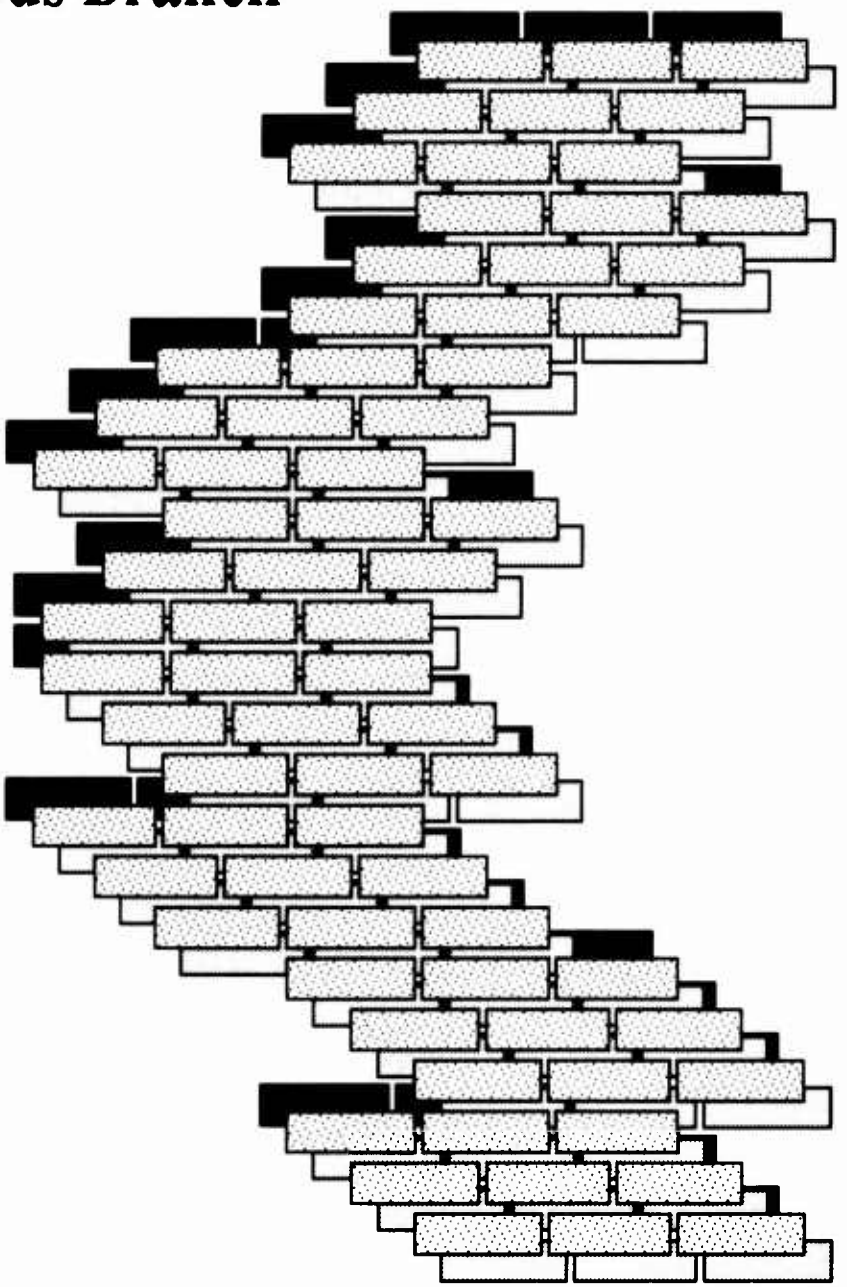
The papers contained in this document were meant to provide the program management staff with an additional means of reviewing the status and progress of extramural and selected in-house studies in the area of new carcinogenicity models. The papers are, therefore, the principal investigators' (PIs') best efforts to characterize the research at the time of the meeting. Some papers cover the results of several years of research and others outline only the planned research of a new project. The data presented and conclusions of the authors represent the best professional judgment of the respective PI at the time of the workshop but are not equivalent to a peer-reviewed report of completed research.

Compendium of the FY1988 & FY1989 Research Reviews for the Research Methods Branch



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Disclaimers

The views, opinions, and/or findings contained in this report are those of the author(s) and should not be construed as official Department of the Army position, policy, or decision, unless so designated by official documentation.

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Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals, and experiments involving animals adhered to the standards stated in the Guide for the Care and Use of Laboratory Animals, NIH Publication 85-23, 1985 edition.

CONTENTS

FOREWORD

Since 1983, the U.S. Army Biomedical Research and Development Laboratory (USABRDL) has conducted a research program focused on the development of new methods for assessing the carcinogenic hazard resulting from exposure to chemicals in the workplace or the environment. This research has been conducted both in-house and extramurally and has involved scientists from other Federal agencies, academic institutions, and the private sector. The need to enhance the integration of the research became increasingly evident as the program grew in the late 1980's. This growth came principally as a result of the U.S. Army Corps of Engineers sponsorship of research to develop environmental applications of new, *in vivo* cancer assessment models for use in the burgeoning Department of Defense environmental remediation program. The convening of an annual research review meeting was undertaken to facilitate the integration and technology transfer of this research program. This publication is a compendium of the first two such meetings.

The papers contained in this document were meant to provide the program management staff with an additional means of reviewing the status and progress of extramural and selected in-house studies in the area of new carcinogenicity models. The papers are, therefore, the principal investigators' (PIs') best efforts to characterize the research at the time of the meeting. Some papers cover the results of several years of research and others outline only the planned research of a new project. The data presented and conclusions of the authors represent the best professional judgment of the respective PI at the time of the workshop but are not equivalent to a peer-reviewed report of completed research.

The scientific review of the program and interaction among investigators accomplished as a result of these workshops were extremely beneficial to the USABRDL staff and participants. I wish to express my gratitude to Ms. Naomi Spalding and Mrs. Bev Smith for the administrative coordination of these workshops. Their efforts were of inestimable value to the success of this project. I am keenly aware that the success of this research program is due to the innovative, conscientious, and unselfish research of the scientists and technicians at USABRDL and our extramural laboratories. I continue to learn from them all and am grateful for their efforts. This research has been supported by the U.S. Army Medical Research and Development Command and the U.S. Army Corps of Engineers. Their continued encouragement has been critical to the success of this program.

Henry S. Gardner, USABRDL

1988 RESEARCH REVIEW MEETING

23 August 1988

HEPATIC ORNITHINE DECARBOXYLASE ACTIVITY IN SMALL FISH

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INTRODUCTION

Ornithine decarboxylase (ODC) is the rate-limiting enzyme in the polyamine biosynthetic pathway. ODC is found in bacteria, plants, invertebrates, and vertebrates and is the only known enzyme that will convert ornithine (by decarboxylation) to putrescine. Putrescine is then converted to spermine and spermidine (Russell, 1971; Russell, 1973; Canellakis et al., 1979). These polyamines are necessary for growth in a variety of cells, but the mechanism of their action is still unclear (Corti et al., 1987; McCann, 1980; Russell, 1973; Tabor and Tabor, 1964).

Trophic hormones, mitogens, carcinogens, and tumor-promoting age...s have been shown to induce ODC activity and, hence, polyamine production (O'Brien, 1976; Olson and Russell, 1979; Russell et al., 1976; Russell, 1980). High levels of ODC are, therefore, found in most tissues undergoing a rapid growth phase, such as embryonic tissue, regenerating tissue, and neoplasms (Manen et al., 1976; Russell, 1985; Russell and Snyder, 1968). A great deal of ODC research has focused on induction of enzyme activity in a target organ or tissue following carcinogen exposure.

Most ODC research has been done with homeothermic animals. The majority of these studies focus on the induction of rat liver ODC with carcinogens such as diethylnitrosamine (DEN) (Olson and Russell, 1979) and 4-dimethylaminoazobenzene (Scalabrino et al., 1978). Induction of ODC in mouse skin with the classical tumor promoter 12-O-tetradecanoylphorbol-13-acetate has also been extensively studied (Mufson et al., 1979; O'Brien, 1976; O'Brien et al., 1975; and Verma et al., 1980). In human cancer patients, ODC has been shown to be useful in some instances as a biochemical tumor marker (Loprinzi et al., 1985; Garewal et al., 1987).

There has been very little research done to date, however, on ODC activity in poikilothermic species. Increased water temperature correlated well with increased ODC activity in loach embryos (Neyfakh et al., 1983). Comparison of ODC activities in the liver and kidneys of sea bass with that of rats showed ODC to be more active but less stable in the

fish (Corti et al., 1987). Winter flounder injected with methylmercury showed increased ODC activity in the liver and kidneys (Manen et al., 1976).

It is the purpose of this research to adapt mammalian methods of detecting ODC to the Japanese medaka (*Oryzias latipes*). The medaka and other small fish species have received extensive attention recently as alternative species in carcinogenicity testing (Hoover, 1984). If a consistent method can be established to detect ODC levels in small fish species, then the next step in the research would be to determine if ODC is indeed inducible in the fish with carcinogens and/or tumor-promoting agents, as is the case with mammalian species. If this were true, then the usefulness of ODC as a mammalian enzyme tumor marker could be extended to poikilothermic species as well and serve as an early warning sign of neoplasm de elopment in fish carcinogenicity studies.

MATERIALS AND METHODS

Adult female medaka, approximately 11 months old and weighing about 0.5 g, were obtained from in-house culture tanks (temperature range 24 to 26°C). To assay for ODC activity and protein levels in the liver, the fish were sacrificed by cervical transection and their livers removed, weighed, and placed on ice in 1.5-ml Eppendorf tubes. The procedure for the ODC assay was adapted from a method by Kozumbo et al. (1983). Usually a pool of three to six livers per tube was needed to obtain enough protein and supernatant for the assays. One hundred µl of buffer containing 50 mM sodium phosphate, 0.1 mM EDTA, and 0.2 mM pyridoxal phosphate was added to each tube. The livers were then subjected to cell disruption by one of two methods: freeze-thawing or sonication. In the freeze-thaw method, the vials containing the livers were placed on dry ice and then thawed in a 37°C water bath. This process was repeated three times. With the sonification method, an ultrasonic processor (Sonics and Materials, Vibra Cell, Model VC-50) was used to disrupt the cells. This method was much quicker than the freeze-thaw method. Protein yields for both methods were comparable, so sonification was preferred.

The disrupted cells were then centrifuged at 12,000 g for 4 minutes to obtain soluble supernatant. Aliquots of the supernatant were frozen at -70°C for subsequent analysis of ODC activity and protein. Protein levels were determined by the Bradford method (Bradford, 1976). ODC activity was determined by a modified procedure from Kozumbo et al. (1983). Briefly, 1,050 µl of liver supernatant were incubated at 37°C for 1 hour in 1.5-ml Eppendorf vials containing 125 nCi of radiolabelled L-[1^{14}C] ornithine hydrochloride (Amersham/Searle, 60 mCi/mmol) in a 50-mM sodium phosphate buffer containing 0.1 mM EDTA, 1.2 mM pyridoxal phosphate, 24 mM dithiothreitol, and 1.96 mM cold ornithine. A drop of 40% KOH

was placed on the underside of the vial cap to trap CO_2 produced from the decarboxylation of ^{14}C ornithine during the incubation. Incubation was terminated by placing the vials in 95°C water for 2 minutes. A Beckman LSC 5701 was used to measure radioactivity trapped in the KOH drop on the vial caps. The ODC activity was expressed as CO_2 evolved/hour/mg protein.

RESULTS AND DISCUSSION

The average medaka liver weighed approximately 6 mg. Protein values obtained ranged from 3 to 4 μg protein/mg liver tissue. ODC levels ranged from 0.03 to 0.44 nmole CO_2/ hour/mg protein. These values were comparable to ODC activity levels in the uninduced sea bass (*Dicentrarchus labrax*) liver (Corti et al., 1987) and loach (*Misgurnus fossilis*) embryos (Nefakh et al., 1983) (see Table 1). In the winter flounder (*Pseudopleuronectes americanus*), a single portal vein injection of 2 mg/kg methyl mercury caused a significant ninefold increase in ODC activity over uninduced fish (Manen et al., 1976).

The next step in this research project will be to attempt to induce ODC in the medaka liver with DEN in the aquarium water. Previous studies in this laboratory have shown a 48-hour dose of 200 mg/liter DEN to be hepatocarcinogenic to the medaka. It is hoped that variability in baseline levels of medaka liver ODC activity (0.03-0.44) will be negligible once the enzyme is induced. The significance of ODC induction in fish and its applicability and importance in cancer hazard assessment also need to be addressed.

Table 1. ODC Activities in Poikilothermic and Homeothermic Animals

Species (Weight)	Organ or Tissue	Treatment	Length of Time to Sacrifice (hr)	ODC Activity, nmole CO_2/hr/mg Protein	Source
Sea bass (210-300 g)	Liver	None	-	0.8	Corti et al., 1987
Winter flounder (200-400 g)	Liver	Saline methyl mercury 2 mg/kg, i.v.	48 48	2.0 18.0	Manen et al., 1976
Loach embryos (*Misgurnus fossilis*)	Entire (early blastula)	None	-	0.02-0.82	Neyfakh et al., 1983
Medaka (0.5 g)	Liver	None	-	0.03-0.44	
Rats (100 g)	Liver	DMSO TPA, 20 μg, i.p.	4 4	0.002 0.120	Bisschop et al., 1981
Rats (130-150 g)	Liver	Saline DEN 200 mg/kg, i.p.	1 1	0.017 0.229	Olson et al., 1979
Mice (20 g)	Skin	Acetone TPA, 17 nmol	4	<0.1 4.8	O'Brien, 1976

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NEW MODELS FOR ONCOGENE ISOLATION IN THE STUDY OF CARCINOGENESIS

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ABSTRACT

The role of activated proto-oncogenes in the etiology of chemically induced tumors in Japanese medaka *(Oryzias latipes)* was examined. Tumors were induced in either medaka or fry by exposure to methylazoxymethanol acetate (MAM-Ac) or diethylnitrosamine (DEN). Tumor and/or liver tissue was excised, a portion preserved for histopathological analysis, and the remainder used for DNA extraction. High-molecular-weight fish DNA was co-transfected with pSV_2neo into mouse NIH 3T3 fibroblasts by calcium phosphate precipitation. The transforming ability of medaka DNA was examined by standard focus assay, nude mouse assay, and colony selection in defined media. DNA isolated from a DEN-induced mesenchymal-type lesion was highly tumorigenic by all criteria, with secondary transfectants causing tumors in nude mice of >20 mm in 1.5 weeks post-injection. Southern blot analysis of restriction digests of these transfect DNAs when hybridized under stringent conditions to medaka genomic DNA probes revealed bands present in tumor-transformed cells. No bands were seen in DNA digests from NIH 3T3 control cells or in cells transfected with DNA from nontumorigenic medaka controls. This suggests the presence of fish-specific sequences in the transformants. These sequences do not appear to be homologous to K-*ras*, H-*ras*, N-*ras*, c-*myc*, m-*met*, *neu*, or *erb*B. Cloning of these presumptive fish oncogenes is now in progress.

INTRODUCTION

Transforming genes arising from normal cellular genes have been detected in a variety of tumors by DNA transfection techniques. Studies of these genes promise insight into normal and pathological mechanisms of growth control. Although work in this area has progressed rapidly in mammalian systems, examination of teleost tumors at the molecular level is just beginning. That certain carcinogens can induce tumors in fish is well documented (Hoover,

1984). Correlation of tumor frequency with high levels of chemical pollutants strongly suggests their involvement in the induction and/or maintenance of naturally occurring fish tumors (Kraybill et al., 1977). Proto-oncogenes have recently been isolated from several fish species (Schartl and Barnekow, 1982; Anders et al., 1984; Van Beneden, et al., 1986; Nemoto et al., 1987; McMahon et al., 1988). Recently, McMahon et al. (1988) have shown activated K-*ras* oncogenes in DNA from liver tumors of winter flounder that are capable of transforming NIH 3T3 cells. Development of transfection systems to isolate transforming sequences from fish neoplasms is an important step toward the development of alternative model systems for the study of tumor cell proliferation.

MATERIALS AND METHODS

Induction of Tumors

Medaka used in these experiments were obtained from in-house medaka culture tanks. Eggs were collected and transferred to 1-liter mesh-bottomed beakers and held until transferred to test solutions. DEN-treated fish were exposed as 14-day-old fry to 200 mg/liter DEN for 48 hours. The fish were not fed during this time. In a second experiment, 8-day-old unhatched embryos were treated with 50 mg/liter MAM-Ac for 4 hours. Water samples were taken at the beginning and end of the exposure periods for analytical verification of the test compound concentrations. After treatment, the fish were rinsed and transferred to holding tanks. Fry were raised for 6 months at 20°C or 25°C. At time of sacrifice, livers (or grossly visible tumors, as in the MAM-Ac treated fish) were removed and a portion preserved in Bouin's fixative for histopathological analysis. The remainder was either used immediately to prepare DNA or frozen in liquid nitrogen and stored at -70°C until used.

DNA Preparation

High-molecular-weight DNA was prepared by quick dounce homogenization. Individual tissues were homogenized in 0.14M NaCl, 0.014M sodium citrate (0.9 x SSC) with 0.57 mg/ml proteinase K, and phenol was extracted immediately. Following extraction with chloroform/isoamyl alcohol (CIA; 24:1) and ethanol precipitation, the sample was resuspended in 0.5 volume of 1 x SSC and sequentially incubated with RNAse A (0.31 mg/ml) and proteinase K (0.31 mg/ml), 30 minutes each, at 37°C. DNA was re-extracted with an equal volume of phenol and CIA, then reprecipitated in ethanol and resuspended in 0.25 volume 0.1 x SSC. DNA concentration was measured by absorbance at 260 nm and integrity examined by

agarose electrophoresis. DNA was isolated from tissue culture cells by a modified Hirt extraction.

Transfection Procedure

In order to identify transforming sequences, high-molecular-weight DNA isolated from fish tumors and from normal tissues (controls) was examined by transfection assay. A stock of NIH 3T3 cells (490 N3T) was obtained from Dr. D. Blair, Frederick Cancer Research Facility, National Cancer Institute. Cells were maintained at levels below confluency in Dulbecco-modified Eagle medium (Gibco) supplemented with 10% fetal calf serum. To each plate of 3 $\times 10^5$ NIH 3T3 cells, 25 μg of fish genomic DNA (prepared as above) was co-transfected with 2 μg of a neomycin-resistant plasmid (pSV_2neo) in the presence of calcium phosphate (Pellier et al., 1980). A total of four plates (100 μg fish DNA) of each sample were tested, which was expected to provide one genomic equivalent of DNA. Cells were grown in the presence of geneticin (G418) for 2 weeks. Drug-resistant colonies were selected, harvested by trypsinization, and replated (in the absence of G418) in a standard focus assay, a colony selection assay, and/or injected into athymic mice.

Standard Focus Assay

G418-selected cells were replated in media without G418, grown to confluency, and examined for morphologically altered cells. Foci were counted, picked, and expanded. DNA was isolated by the Hirt extraction procedure.

Colony-Selection Assay

Cells from the same pool were replated in minimal media (QBSF or QBSF supplemented with 0.1% serum; Quality Biologicals). Cells that formed colonies were counted and DNA isolated as above.

Nude-Mouse Assay

The remaining G418-selected cells were injected into athymic mice (1.5×10^6 cells/mouse). Mice were examined for tumors at the site of injection (positive results usually occurred $\leq$ 6-8 weeks). Tumors were excised and portions frozen for DNA extraction and/or diced and placed in media for growth of tumor explants.

Growth in Soft Agar

In order to examine cells for anchorage independence, cells from foci were examined for their ability to grow in soft agar (0.23%).

Identification of Transforming Sequences

DNA isolated from transfected cells was digested with restriction enzymes, electrophoresed on agarose gels, transferred to nitrocellulose, and the resulting Southern blots (Southern, 1975) were hybridized to nick-translated oncogene sequences. Fragments used as hybridization probes included v-*erb*B, H-*ras*, v-*myb*, v-*abl*, v-*erb*A, v-*sis*, and v-*src* (all from Oncor Science); c-*myc* (rainbow trout); v-*ets* (fragment E1.2B, Watson et al., 1985); m-*met* (obtained from T. Kmieck); and *neu* (obtained from M. Barbacid). To identify sequences unique to medaka, Southern blots were hybridized to medaka genomic DNA under stringent conditions (50% formamide; 42°C).

RESULTS

Pathological evaluation of the tissue samples (see Table 1) revealed a malignant, very aggressive rhabdomyosarcoma in the tissue taken from the MAM-Ac-treated fish. The majority of the livers excised from fish treated with DEN had a toxic appearance. A single fish liver, MD6-14, contained a very aggressive cholangiocarcinoma.

DNA from these tissues was examined by primary transfection assay. DNA isolated from the MAM-Ac-induced rhabdomyosarcoma had only a marginal effect on transformation of NIH 3T3 cells. The transforming ability of DNA isolated from the DEN-exposed animals appeared to correlate with the degree of malignancy observed in the liver. DNA isolated from the cholangiocarcinoma lesion in MD6-14 was the most tumorigenic in the assay systems. The nude mouse assay showed very high background in this particular experiment. Subsequent investigation revealed that this may have been due to higher sensitivity of a new stock of athymic mice.

Cells isolated from individual foci were tested for anchorage independence by their ability to grow in soft agar, a characteristic of transformed cells. Table 2 shows that cells isolated from plates transfected with MD6-14 DNA grew as well as control cells.

DNA extracted from either individual foci or soft-agar colonies was used in a second round of transfection. DNA isolated from the cholangiocarcinoma lesion of MD6-14 very rapidly transformed NIH 3T3 cells as determined by standard focus assay, colony selection in minimal media (QBSF), and the nude mouse assay. Tumors in nude mice grew to 20 mm after

Table 1. Histopathological Analysis of Medaka Tissue*

Fish	Pathology
MD-Rh	rhabdomyosarcoma, very aggressive, malignant
MD6-12	toxic (bile duct ectasia)
MD6-13	bile duct hyperplasia
MD6-14	cholangiocarcinoma
MD6-15	toxic (spongiosis hepatis, hepatocellular vacuolation)
MD6-17	normal (macrophage aggregates)
MD8-12	normal (hepatocellular vacuolation)
MD8-13	normal (hepatocellular vacuolation)
MD8-14	bile duct hyperplasia
MD8-17	normal (hepatocellular vacuolation)
MD8-18	hepatocellular carcinoma

*Liver tissue was examined in all fish except MD-Rh, which has a visible tumor of striated muscle.

only 1.5 weeks. DNA isolated from the MAM-Ac-induced rhabdomyosarcoma had only a marginal ability to transform NIH 3T3 cells.

Hybridization of Southern blots of EcoRI and BamHI digests of DNA from two secondary transfectants (induced by MD6-14 DNA) to medaka genomic sequences under conditions of high stringency revealed specific bands (data not shown). These bands were not present in DNA isolated from NIH 3T3 cells alone or in DNA from cells transfected by nontumorigenic medaka DNA. This suggests that the transformation of the NIH 3T3 cells is due to specific fish sequences. These sequences have not yet been identified, but do not appear to be homologous to H-*ras*, N-*ras*, K-*ras*, v-*erb*B, *myc*, *neu*, or m-*met* as determined by Southern blot hybridization.

DISCUSSION

A transfection assay has been developed that enables the screening of chemically induced tumors in the medaka for presumptive activated oncogene sequences. The transforming ability of DNA isolated from DEN-treated fish appears to be correlated with the degree of malignancy. DNA from the very aggressive, cholangiocarcinoma gave the highest efficiency of transformation of those examined in this study. However, DNA from the MAM-Ac-induced malignant rhabdomyosarcoma was only marginally effective in the transfection system. These findings suggest that perhaps a "transforming gene," if present in the tumor, does not function effectively in the transformation of NIH 3T3 cells.

Table 2. Growth of Cells Isolated from Individual Foci in Soft Agar

Plate Number	Number of Colonies*
TR8-1A1	-
TR8-1A3	-
TR8-2A1	-
TR8-2A3	-
TR8-5C1	-
TR8-5D2	-
TR8-6B2	+++
TR8-6D1	+
TR8-8C1	+
TR8-8C2	+
TR8-8D2	+
TR8-11B1	+
TR8-11B3	-
TR8-12C1	++
TR8-12C3	++++
TR8-13A2	+
TR8-13B1	++++
TR8-14A2	-
TR8-17D1	-
TR8-18A3	-
TR8-19B2	-
TR8-20A2	-
TR8-20B1	-
TR8-21C2	+
TR8-21D3	+
TR8-22C2	-
TR8-23A1	-
TR8-24A1	-
TR8-25A1	-
TR8-25A2	-
TR8-27B2	-
TR8-29C2	+
TR8-29C3	+
ras-transfected control	++++

*Number and size of colonies are relative to *ras*-transfected positive control (-, negative; +, meaning least number of colonies to ++++, most number of colonies).

The authors conclude that the transformation induced by MD6-14 DNA is due to specific sequences in the fish tumor DNA because: (1) specific bands hybridizing to medaka sequences are present in transformed cells from primary, secondary, and tertiary transfectants that are absent in NIH 3T3 cells alone and NIH 3T3 cells transfected with control medaka DNA; (2) two separate primary transfection events using DNA isolated from the mesenchymal lesion had high transforming ability; and (3) the transforming efficiency increased in primary, secondary, and tertiary (data not shown) transfections.

By Southern analysis, the transforming sequence does not appear to be homologous to any of the known oncogenes tested. Experiments are currently underway to isolate, clone, and sequence this presumptive fish-transforming sequence in order to determine its identity.

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ULTRASTRUCTURAL CHARACTERIZATION OF SELECTED CHEMICALLY INDUCED LESIONS IN THE MEDAKA*

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ABSTRACT

The primary objective of this investigation was to determine the ultrastructural effects on fry and embryos of the Japanese medaka, *Oryzias latipes*, after an acute exposure to diethylnitrosamine (DEN). In one phase of the study, 14-day-old fry were exposed to 200 mg/liter DEN for 48 hours and then held in clean water at 25°C. Thirteen weeks subsequent to the exposure, four fish were removed from the tank, and their livers were excised and processed for electron microscopy. The livers displayed isolated islands of altered hepatocytes characterized by markedly swollen mitochondria, distended cisternae of rough endoplasmic reticulum, and dissociated nucleoli. Pronounced clear zones, void of intact cells, circumscribed blood vessels. Cellular debris from degraded hepatocytes collected in the space. A proliferated mass of what appeared to be bile preductular cells was evident in the exposed liver.

During the second phase of the study, a group of embryos was treated continuously with 3,000 mg/liter DEN for 48 hours. The embryos were rinsed and held in clean water at 30°C. Fifty weeks subsequent to the exposure, six fish were sacrificed and the livers and kidneys were excised and processed for electron microscopy. The kidneys displayed several manifestations of cytotoxicity, including the degradation of epithelial cells and dilation of proximal tubules as a result of the accumulation of cellular debris within the lumen. The livers presented large cyst-like zones with a structural framework comprised of perisinusoidal-like cells. Remnants of necrotic cells were observed in many of the cystic zones. Clusters of macrophage-like cells tended to concentrate in regions adjacent to the cysts.

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*The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

INTRODUCTION

Selective species of fish have proven valuable as independent models for carcinogenicity studies and as complementary extensions of current mammalian testing systems (Hoover, 1984). The medaka (*Oryzias latipes*), a small killifish native to Japan, has served as a popular model for investigations involving DEN, a site-specific carcinogen for hepatic neoplasms (Norton and Gardner, 1987; Kyono, 1978). The majority of carcinogenicity studies concerning chemical induction in fish models have concentrated on the liver (Couch and Courtney, 1987; Hinton et al., 1984), an organ which possesses the oxidative enzyme systems that are necessary for the bioactivation of many carcinogens, including DEN (Stegeman, 1981).

Although valuable information regarding the characterization and classification of chemically induced tumors in fish has been acquired (Hawkins et al., 1986; Aoki and Matsudaira, 1984; Hendricks et al., 1980), there are still notable voids in knowledge of the biochemical and ultrastructural changes that precede cellular transformation. The primary objective of these investigations was to determine the ultrastructural effects of an acute exposure to DEN on fry and embryos of the Japanese medaka. Hepatic and renal tissues were analyzed for evidence of cytotoxicity and/or cellular lesions.

MATERIALS AND METHODS

A group of 14-day-old medaka fry was selected from a stock population and exposed to DEN at a concentration of 200 mg/liter for 48 hours. The fish were rinsed and held in clean water at 25°C. An additional group of fry was maintained at 25°C and functioned as a control group. At 13 weeks subsequent to the exposure, four fish from each tank were removed and the livers excised. Sections of the livers were minced and placed in a cold solution of 4% glutaraldehyde, which was buffered with 0.1M sodium cacodylate (pH 7.4). After 2 hours of fixation, the tissues were rinsed several times in buffer and post-fixed in 2% osmium tetroxide for 1 hour. The samples were rinsed in buffer, dehydrated in a graded series of ethanol, and embedded in Epon 812. Thin sections were cut by an ultramicrotome, stained with lead citrate and uranyl acetate, and examined with a JEOL 100B transmission electron microscope.

Exposure of Embryos

A group of embryos was treated continuously with 3,000 mg/liter of DEN for 48 hours. The embryos were rinsed and held in clean water at 30°C. An additional group was maintained at 30°C and served as a control. At 50 weeks subsequent to the exposure, six fish from each tank were removed and the livers and kidneys excised. Sections of tissue from each organ were minced and fixed in a cold solution of 4% glutaraldehyde, which was buffered

with 0.1M sodium cacodylate (pH 7.4). After 2 hours of fixation, the tissues were rinsed in buffer and post-fixed for 1 hour in 2% osmium tetroxide. The tissues were rinsed in buffer, dehydrated in a graded series of ethanol, and embedded in Epon 812. Thin sections were cut, stained, and observed with a JEOL 100B transmission electron microscope. Selective tissue was also processed for a periodic acid-Schiff reaction.

RESULTS

Exposure of Fry

The majority of hepatic parenchymal cells displayed no discernible effects of exposure. However, isolated hepatocytes were characterized by various manifestations of cytotoxicity. Distinct ultrastructural alterations, including dissociation of the outer nucleolonema which resulted in a localized dispersion of the granular elements, were noted among nucleoli of compromised cells. Dilated cisternae of rough endoplasmic reticulum and markedly swollen mitochondria that contained vesiculated cristae were characteristic features of compromised hepatocytes (Figure 1). Also evident were mitochondria in a state of autolysis as manifested by the presence of myeloid figures in continuum with the organelle's outer membrane.

Pronounced cystic zones were observed in association with blood vessels of exposed tissue. Cellular debris from degraded hepatocytes collected in the cyst. Hepatocytes adjacent to the cystic zone displayed definitive features of cytotoxicity, including fragmented membranes and swollen mitochondria (Figure 2).

Several distinct regions of exposed liver were characterized by a population of what appeared, ultrastructurally, to be bile preductular cells. Desmosomes were observed between adjacent preductular cells; however, no junctions were noted in the interface between hepatocytes and preductular cells. Several individual hepatocytes bordering the preductular array appeared to be enveloped by single preductular cells. Electron-dense material permeated intercellular spaces between adjacent hepatocytes and preductular cells. Small uncoated vesicles containing the material were evident along the plasma membrane of preductular cells (Figure 3).

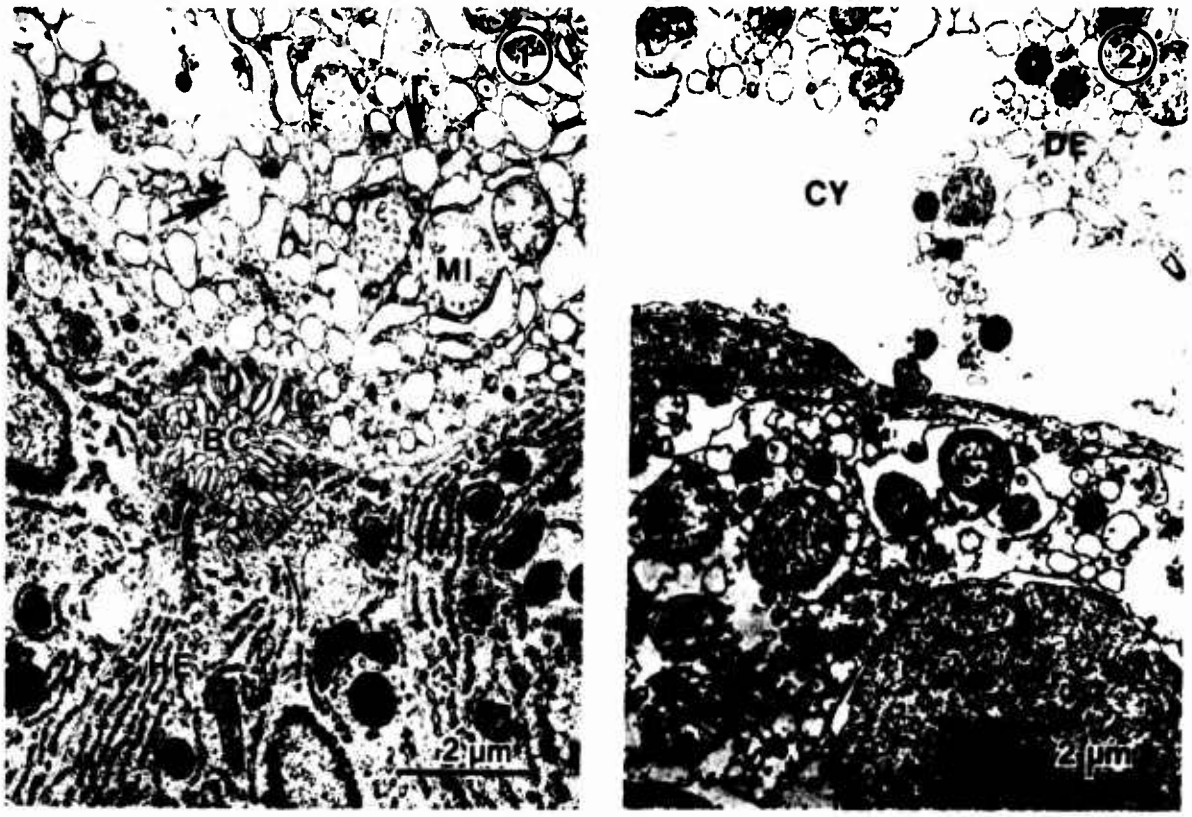

Figure 1. Compromised hepatocyte displays swollen mitochondria (MI) and distended cisternae of rough endoplasmic reticulum (arrows). Adjacent hepatocytes (HE) and bile canaliculus (BC) appear normal. Exposed liver.

Figure 2. Necrotic hepatocyte contains swollen mitochondria (MI), distended cisternae of rough endoplasmic reticulum (arrow), and fragmented nuclear membrane (arrowhead). Cystic zone (CY) contains cellular debris (DE). Exposed liver.

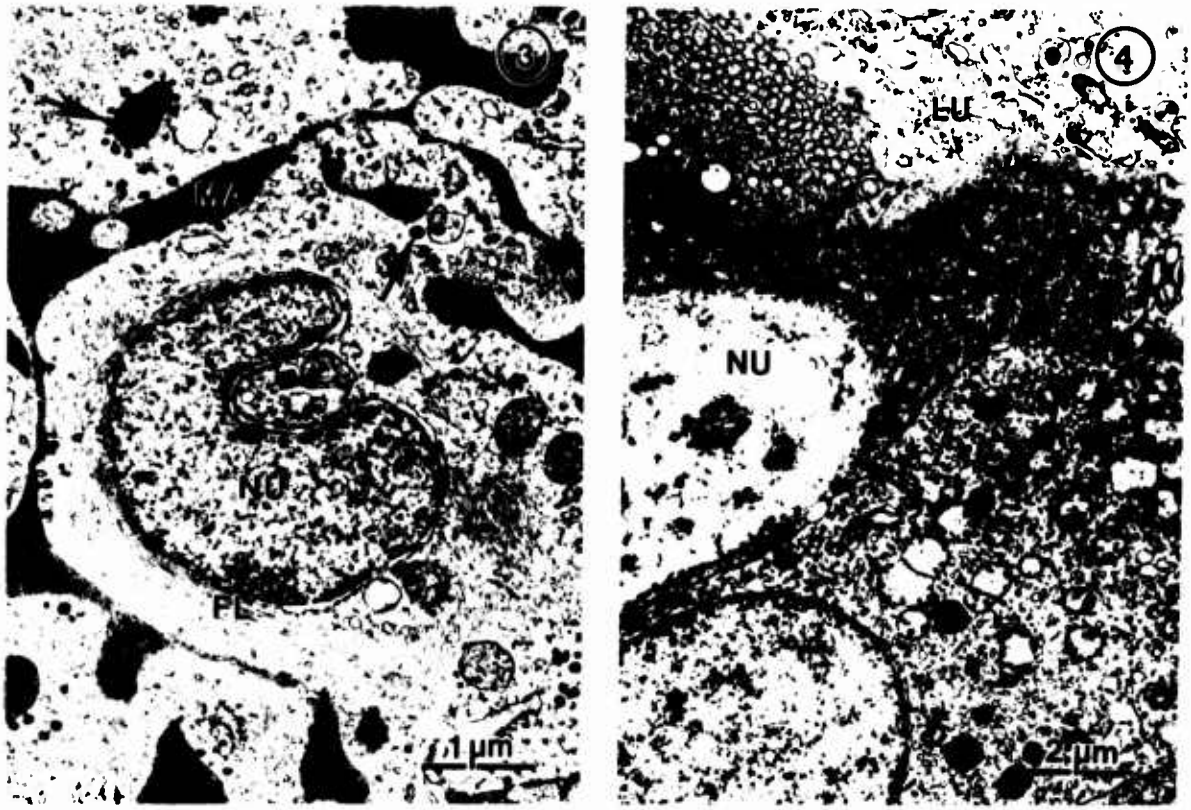

Figure 3. Bile preductular cell is characterized by the presence of intermediate filaments (FL) and indented nucleus (NU). Electron-dense material (MA) collects within intercellular spaces and is evident in cytoplasmic vesicles (arrows). Exposed liver.

Figure 4. Lysed epithelial cell (EP) of proximal convoluted tubule releases contents into tubular lumen (LU). The nucleus (NU) contains clusters of electron-dense material and lacks an outer envelope (arrow). Exposed kidney.

Exposure of Embryos

Kidney

Several of the proximal convoluted tubules examined appeared markedly distended as a result of the substantial accumulation of granular and flocculent material, myeloid figures, and isolated organelles within the lumen. Epithelial cells lining the tubular lumen displayed large apical blebs or fragmented plasma membranes, which resulted in the release of cytoplasmic contents into the lumen (Figure 4). Cells that demonstrated irreversible traits of cytotoxicity possessed pyknotic nuclei and swollen mitochondria with a clear matrix and fragmented outer membrane (Figure 5).

Pronounced aberrations of cellular surface projections included the dissociation and occasional fusion of the plasma membranes that normally form the outer boundary of microvilli and cilia. Microvilli shortened significantly and increased in width to length ratio by a factor greater than three. The basement membrane juxtapositioned to epithelial cells of the proximal tubule was characterized by the presence of loose cohesive material and short fibrous strands. The sole ultrastructural change detected within the glomerular complex was a slightly thickened or disrupted basement membrane composed of closely opposed laminar material.

Liver

Clusters of macrophage-like cells, which presented a positive periodic acid-Schiff reaction, tended to populate various regions of the exposed liver. The cells were characterized by a low electron-dense cytoplasm and relatively few organelles (Figure 6). A majority of the cells possessed Mallory bodies, well-developed Golgi complexes, and relatively few cisternae of rough endoplasmic reticulum. Many of the hepatocytes associated with the array of macrophage-like cells were pleomorphic in appearance, contained dilated cisternae, and tended to concentrate lipid inclusions.

Large cystic lesions were evident in several regions of the liver (Figures 7 and 8). Cells forming the framework of the cysts featured ultrastructural characters that were similar to those of the perisinusoidal cells associated with the space of Disse. The cells contained elongated, irregular nuclei; abundant quantities of pinocytotic vesicles; clusters of intermediate filaments; and relatively few organelles. Cytoplasmic processes that formed partitions of the compartmentalized cysts were maintained in position by desmosomes. Cavities of the cystic lesions varied with regard to their content and size. Some were completely void of material or contained remnants of necrotic cells, while others possessed lymphocytes, macrophages, and cells of an unknown origin.

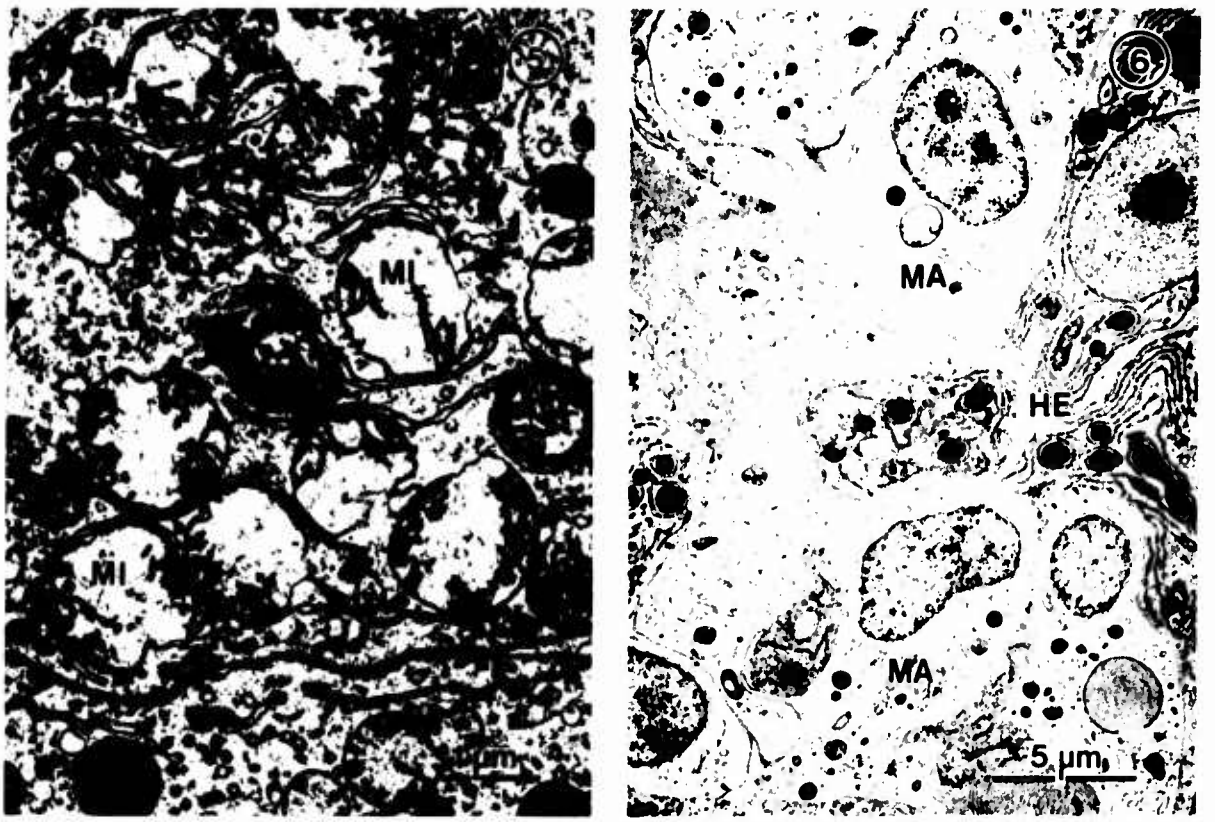

Figure 5. Epithelial cell of proximal tubule illustrates swollen mitochondria (MI), which possess a clear matrix and fragmented outer membrane (arrows). Exposed kidney.

Figure 6. Macrophage-like cells (MA), characterized by an electron-lucent cytoplasm and relatively few organelles, are interspersed among hepatocytes (HE). Exposed liver.

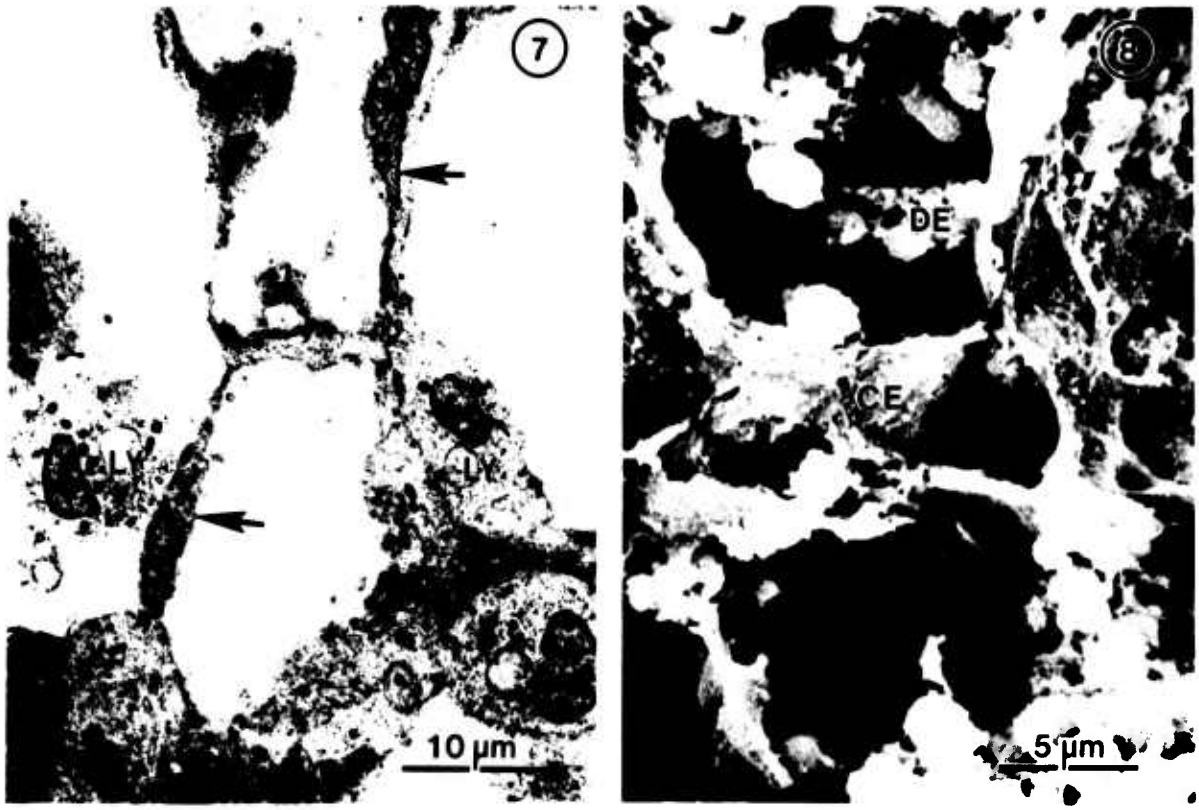

Figure 7. Walls of compartmentalized cysts are comprised of intact elongated cells (arrows), while many of the cells within the cysts are lysed (LY). Exposed liver.

Figure 8. Scanning electron micrograph of cystic lesion reveals portions of cellular debris (DE) within the cavities and squamosal nature of cells (CE) forming the framework. Exposed liver.

DISCUSSION

Results from the two investigations indicate that acute exposure to DEN by either medaka fry or embryos elicits a variety of ultrastructural aberrations. Several cell types, including parenchymal hepatocytes, bile preductular cells, and perisinusoidal cells, are affected adversely. The general overall manifestations of induced toxicity appear to be consistent with reported pathways of cytotoxic injury (Cheville, 1976). The presence of distended cisternae of rough endoplasmic reticulum and vesiculated mitochondrial cristae represents reversible traits of toxicity. However, the more prevalent forms of cellular injury, including extensive swelling of mitochondria, fragmentation of mitochondrial membranes, and cellular necrosis, indicate irreversible lesions. Certain reversible ultrastructural changes may precede cellular transformation and could, in fact, represent biochemical aberrations that are necessary for the initiation and subsequent development of a neoplastic lesion.

The apparent proliferation and succeeding intimate association of bile preductular cells with hepatocytes does not reflect toxicity at the level of cellular organelles. Although the cells associated with the proliferated mass of preductular elements were similar in ultrastructural composition to those of control tissue, this does not preclude the possible transformation of comparable cell types under similar conditions. In fact, it is not an uncommon occurrence in teleosts to find tumors with both hepatocellular and biliary epithelial constituents (Aoki and Matsudaira, 1977; Ishikawa et al., 1975).

The cyst-like lesions evident in some experimental livers demonstrate certain ultrastructural features that are characteristic of spongiosis hepatis, a specific pathologic condition that has been described in the rat (Bannasch et al., 1981). In both the rat and medaka, the areas of spongiosis appear as multiocular, cyst-like complexes with cytoplasmic processes of morphologically distinct cells forming the walls of individual compartments. Bannasch et al. (1981) have postulated that spongiosis hepatis in the rat is due to a specific alteration of perisinusoidal liver cells, which may be the result of a direct interaction of the carcinogen with the latter cell type.

The spongiosis hepatis detected in this investigation differs in several aspects from the comparable lesion described for the rat (Bannasch et al., 1981). Unlike the mammalian system, there are no discernible collagen fibers or basement membrane-like materials associated with the cells that comprise the walls of the cystic complex in medaka. The ultrastructural discrepancies, regarding protein products of gene activation, which are evident in the two systems may reflect different binding sites on DNA by the chemically modified DEN. Specific ultrastructural features concerning nuclear morphology, heterochromatin to

euchromatin ratios, and intermediate filaments tend to indicate that perisinusoidal cells represent the cellular form from which the lesion evolved.

DEN is recognized primarily as a site-specific carcinogen of the liver for fish (Khudoley, 1984). However, related compounds such as dimethylnitrosamine have proven to be potent inducers of tumors in a variety of organs including the kidney (Hard et al., 1984). Renal tissue analyzed in this investigation displayed several indicators of severe cytotoxicity, including focal necrosis of cells comprising various regions of the proximal convoluted tubule. The extensive amount of debris concentrated within the proximal tubule appears to be derived from necrotic cells that lyse and release their contents into the tubular lumen. Alterations regarding microvilli and the plasma membrane of epithelial cells contacting the lumen are particularly evident. The ultrastructural manifestations of toxicity described in this paper have not been reported for any fish models employed in carcinogenicity studies.

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DEVELOPMENT OF AN AQUATIC BIOASSAY FOR CARCINOGENICITY AND TOXICITY TESTING USING THE MEDAKA (*Oryzias latipes*) AS A MODEL

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INTRODUCTION

Environmental contamination with various carcinogenic and toxic compounds has led to massive research efforts to determine human risk involved in exposure to these substances. The use of animal models to study this risk has come about because known carcinogens for humans are also carcinogenic for animals, and the morphology and behavior of certain types of carcinogen-induced neoplasms in humans are similar to those in animals (Farber, 1976). One of the most important sources of exposure is waterborne contamination, as water in the form of rain, lakes, and oceans is a tremendous disseminator of substances. The level of environmental pollution in the United States is great, and an effect on fish has been evidenced by a high prevalence of neoplasms in certain fish in various locations around the United States (Harshbarger, 1984).

This observation has led to the suggestion that small fish species might be used as alternatives to small mammals in carcinogenicity and toxicity testing bioassay systems. This model, when developed, can be used by the military as a risk assessment to rapidly and accurately evaluate the environmental hazards to field military personnel of contaminated water and/or food supplies by potentially noxious chemicals. In addition, this model can be used by the military to determine environmental safety procedures and human health risks for compounds that might be generated by military operational procedures, and to determine potentially damaging effects of these compounds to aquatic ecosystems.

The small fish bioassay is attractive because of the reduced time requirement for testing compared to rodent studies which can take up to 2.5 years, the ability to use large numbers of small fish, and the decreased expense of maintaining the animals and administering the test substances. With an aquatic test system that includes proper control of light, temperature, and salinity, large numbers of compounds can be tested with relative ease. The short reproductive cycle of the small fish also allows assessment of effects on embryonic, immature, and adult stages of life. However, for this small fish bioassay to be useful, the following objectives need to be met.

OBJECTIVES AND METHODS

Response of Fish Tissue to Injury; Exposure to Carcinogens

For the use of small fish bioassays to be valid, degenerative, toxic, and neoplastic changes in fish tissues must be documented and compared to those seen in rodents and humans. In this research, a batch of medaka was exposed at 2 weeks of age to three levels (100, 200, and 400 mg/liter) of the known carcinogen, diethylnitrosamine (DEN), and sequentially sacrificed every 2 weeks for a period of 3 months, with a final sacrifice at 6 months. Fish were embedded whole in methacrylate for histology and/or liver tissue was processed for electron microscopy. Hepatocellular changes will be documented and compared to those seen after carcinogenic exposure in rodents. Special stains will be employed when deemed necessary to identify cell types. The National Toxicology Program's current nomenclature for hepatoproliferative lesions in rats includes: (1) foci of cellular alteration (clear, eosinophilic, basophilic, and mixed foci); (2) hepatocellular adenoma; (3) hepatocellular carcinoma; and (4) hyperplasia. Additional changes seen in rat livers include fatty change, vascular and bile duct ectasia, cystic degeneration (spongiosis hepatis), and cholangiofibrosis (Institute of Laboratory Animal Resources, 1984; Marenpot et al., 1986). All degenerative and neoplastic changes in each group will be graded for the severity of change, as according to Dr. Robert Squire (1984). Table 1 shows the grading scale to be used.

Table 1. Grading Table*

Grade	Degenerative Change	Benign Tumor	Malignant Tumor
1	Minimal		
2	Minimal to moderate	Graded 1-5	Graded 1-5
3	Moderate	depending on	depending on anaplasia,
4	Moderate to severe	size	invasion, and
5	Severe		number of mitoses, etc.

*From Squire (1984).

Hepatocellular lesions will also be documented ultrastructurally to determine pathogenesis and progression of lesions in the medaka. Autoradiography is also being used to document proliferative foci in the liver and to correlate these foci with histologic lesions.

Normal Hepatic Development in the Medaka

The liver is one of the primary organs monitored during carcinogenicity or toxicity testing. However, the fish liver is structurally different from that of mammals, and all of the cell types present in the fish have not been clearly identified and defined. In addition, because tumorigenesis can mimic embryonic and fetal cellular development in a retrograde fashion, and the sequential normal differentiation of these populations has not been adequately described for fish, this research will document the embryogenesis and organogenesis of the medaka liver. Using histologic, ultrastructural, and morphometric techniques, the sequential development of various hepatic cell types from embryonic to adult life will be documented to establish baseline criteria for accurate identification of tumors that may develop due to chemical exposure. Comparisons to mammalian hepatocellular development and differentiation will also be made and similarities and/or differences determined so that responsible, valid conclusions can be made about extrapolating the tissue responses of the fish to those which might occur in humans under similar exposure conditions.

The successful completion of the goals of this research will produce a significant database toward establishing the small fish bioassay as an alternative to mammalian carcinogenicity testing.

PROGRESS TO DATE

All fish have been collected as shown in Tables 2 and 3; only one group in Part A remains to be collected at the 6-month sacrifice. Preliminary data show early normal hepatic development consisting of a bland assortment of glycogen-filled hepatocytes with minimal biliary tract development. DEN exposure produced hepatocellular necrosis, megalocytosis, increased lysosomes, and unusual nuclear inclusions in high-dose groups sacrificed at 17 and 31 days postexposure.

Table 2. Collection of Fish, Part A

Group	Received Fish	Days PE[1]	Total # Fish	Process TEM[2]	Process JB4[3]	Process Autoradiography
I	6/6/88	17	10	6/7/88		
	6/20/88	31	15	6/22/88		
	7/5/88	46	15	7/6/88		
	7/18/88	59	15	7/20/88		
	8/22/88	94	15	9/1/88		
II	6/6/88	17	15	6/7/88		7/5/88
	6/20/88	31	27	6/22/88		7/19/88
	7/5/88	46	27	7/6/88		8/2/88
	7/18/88	59	27	7/20/88		8/12/88
	8/22/88	94	27	9/1/88		9/1/88
III	6/6/88	17	10	6/7/88	7/21/88	
	6/20/88	31	15	6/22/88	7/14/88	
	7/5/88	46	15	7/6/88	7/14/88	
	7/18/88	59	15	7/20/88	7/21/88	
	8/22/88	94	15	9/1/88		
IV	6/6/88	17	10	6/7/88	7/21/88	
	6/20/88	31	15	6/22/88	7/14/88	
	7/5/88	46	15	7/6/88	7/14/88	
	7/18/88	59	15	7/20/88	7/21/88	
	8/22/88	94	15	9/1/88		

[1]PE = Post-exposure
[2]TEM = Transmission electron microscopy
[3]JB4 = Methacrylate embedment for histology

Table 3. Collection of Fish, Part B

Age	ID	GLUT	Bouin's	Received	Process TEM[3]	Process JB4[4]
DY 4E[1]	M88-121-1	1-10	11-20	5/3/88	5/9/88	
DY 6E	M88-123-2	1-10	11-20	5/6/88	5/9/88	5/23/88
DY 8E	M88-125-3	1-10	11-20	5/6/88	5/9/88	5/17/88
DY OP[2]	M88-126-4	1-10	11-20	5/9/88	5/9/88	5/17/88
DY 2P	M88-128-5	1-10	11-20	5/9/88	5/16/88	5/17/88
DY 4P	M88-130-6	1-10	11-20	5/12/88	5/16/88	6/17/88
DY 6P	M88-132-7	1-10	11-20	5/12/88	5/16/88	6/17/88
DY 8P	M88-134-8	1-10	11-20	5/18/88	5/23/88	6/17/88
DY 11P	M88-137-9	1-10	11-20	5/18/88	5/23/88	6/17/88
DY 18P	M88-144-10	1-10	11-20	5/25/88	6/7/88	6/3/88
DY 32P	M88-158-11	1-10	11-20	6/10/88	7/7/88	
DY 61P	M88-188-12	1-10	11-20	7/11/88	8/8/88	

[1]E = Embryo
[2]P = Post-hatch
[3]JB4 = Methacrylate embedment for histology
[4]TEM = Transmission electron microscopy

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FISH AS A PREDICTIVE MODEL FOR EPIGENETIC CARCINOGENS

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BACKGROUND

The majority of known carcinogens display mutagenic potential and cause DNA damage and chromosomal alterations via genotoxic mechanisms. However, there is an emerging new class of chemical carcinogens that do not directly affect DNA and are believed to be nonmutagenic in short-term tests. These chemicals have been designated as epigenetic carcinogens (Thorpe, 1982).

Because short-term mutagenicity tests have not been able to identify epigenetic carcinogens, it has become important to identify specific chemical, physical, and biological properties of known nonmutagenic carcinogens. One such predictive property is the induction of peroxisome proliferation in rodent hepatic parenchymal cells (Reddy and Lalwani, 1983). The peroxisome, which is ubiquitous in plant and animal cells (including fish), is a cytoplasmic organelle characterized by a single limiting membrane and a finely granular or homogeneous matrix. Peroxisomes have been associated with various functions such as gluconeogenesis, lipid metabolism, and detoxification of H_2O_2 (Reddy and Lalwani, 1983).

Hepatic peroxisome proliferation is believed to be very common and an extensive phenomenon in rodents. In addition, studies have shown that it occurs in the livers of species besides rodents such as birds, rabbits, cats, and primates, including humans (Svoboda et al., 1967; Reddy et al., 1982; Hanefeld et al., 1980). Unfortunately, a comprehensive database on interspecies differences in sensitivity to peroxisome proliferation does not exist. While limited interspecies comparisons for peroxisome proliferation exist for mice, rats, hamsters, dogs, and monkeys (Reddy and Lalwani, 1983), no data have been published on fish species.

In rodents, peroxisome proliferation begins within 2 hours of the first dose of the proliferator and reaches a plateau at 14 days. This plateau of proliferation is maintained as long as the agent is administered. There is a rapid decrease in peroxisome density after removal of the agent, and by about 2 weeks the number of peroxisomes returns to normal (Moody and Reddy, 1978).

A number of substances, especially those used as hypolipidemic agents (e.g., clofibrate), have been identified as peroxisome proliferators (Table 1). In addition, an emerging number of broadly distributed environmental contaminants such as plasticizers [e.g., di-2-ethylhexylphthalate (DEHP)] (Moody and Reddy, 1978) and solvents [e.g., trichloroethylene (TCE)] (Elcombe, 1985) have been found to be effective peroxisome proliferators.

Many peroxisome proliferative agents, though hepatocarcinogenic in mice and rats (Table 2), have been shown to be nonmutagenic in a wide range of genotoxic assays. Reddy and Lalwani (1983) have theorized that increased peroxisomal oxidation of fatty acids leads to the formation of increased steady-state concentrations of H_2O_2 which, in turn, may damage cellular components including DNA. During peroxisome proliferation, H_2O_2 production markedly increases, but the capacity for H_2O_2 detoxification via catalase activity remains basically unchanged (Fahl et al., 1984). This shifts the oxidant-antioxidant balance to greatly favor the occurrence of oxidant stress, which could then lead to mutational events and eventually cancer, based on the classical somatic mutation theory of carcinogenesis. In fact, peroxisomes from rodent livers treated with peroxisome proliferators have been found to produce 30 to 70 times more H_2O_2 than unexposed control group peroxisomes (Fahl et al., 1984).

It is important to note that the gross histological and ultrastructural characteristics of hepatocellular tumors induced by carcinogenic peroxisome proliferators, including the hypolipidemic drugs and phthalate ester plasticizers, are multiple and indistinguishable from those induced by well-characterized synthetic and naturally occurring chemical carcinogens (Reddy and Lalwani, 1983). The liver tumors induced by peroxisome proliferators metastasize to the lungs and have been successfully transplanted in syngeneic hosts. Electron microscopic analysis has revealed that the general biological features of hepatocellular neoplasms caused by treatment with carcinogenic peroxisome proliferators are fundamentally similar to those of liver tumors induced by other well-studied liver carcinogens with the exception of a mild to moderate increase in peroxisome numbers (Reddy and Lalwani, 1983). Further, the nongenotoxic liver carcinogen, clofibrate, enhances the hepatotoxicity of previously administered diethylnitrosamine in rodents (Reddy and Rao, 1978; Mochizuki, 1982).

HYPOTHESIS

Fish are beginning to be used more widely as animal models for cancer bioassays. To date, these efforts have focused on the use of fish models to predict the effects of genotoxic

Table 1. Hepatic Peroxisome Proliferators

Common Name	
	Group A - Clofibrate and Its Structural Analogs
Clofibrate	Ethyl p-chlorophenoxyisobutyrate
Nafenopin	2-Methyl-2-(p-1,2,3,4-tetrahydro-1-naphthyl)phenoxylpropionic acid
Methyl Clofenapate	Methyl-2-[4-(chlorophenyl)phenoxy-2-methyl]propionate
Sal-42,348	1-Methyl-4-iperidyl-bis(p-chlorophenoxy)acetate
S-8527	1,1-bis[4'-(1"-Carboxy-1"-methylpropoxy)phenyl]cyclohexane
AT-308	3-[4-(1-Ethoxycarbonyl-1-methylethoxy)phenyl]5-(3-pyridyl) 1,2,4-oxadiozole
Gemfibrozil	5-2[2,5-Dimethylphenoxy]2-2-dimethylpropionic acid
Bezafibrate	2-[4-(2-[4-Chlorobenzamide]ethyl)phenoxy]2-methyl propionate
Fenofibrate	Isopropyl-[4-(p-chlorobenzoyl)-2-phenoxy-2-methyl]propionate
LS-2265	Taurine derivative of fenofibrate
Ciprofibrate	2-[4-(2,2-Dichlorocyclopropyl)phenoxy]-2-methyl propionic acid
Simfibrate	1,3-Propanediol-bis(2-p-chlorophenoxy)-isobutyrate
	Group B - Compounds Structurally Unrelated to Clofibrate
Tibric Acid	2-Chloro-5-(3,5-dimethylpiperidinosulphonyl)benzoic acid
Wy-14,643	[4-Chloro-6-(2,3-xylidino)2-pyrimidinylthio]acetic acid
BR-931	4-Chloro-6-(2,3-xylidino)2-pyrimidinylthio(N-hydroxyethyl) acetamide
Tiadenol	Bis[hydroxyethylthio]7,10-decane
RMI-14,514	[5-Tetracycloxy]2-furan carboxylic acid
DG-5685	5-[4-Phenoxybenzyl]trans-2-(3-pyridyl)2,3-dioxane
DH-6463	5-[4-Phenoxybenzyl]trans-2-(3-pyrimidinyl)-1,3-dioxane
LK-903	α-Methyl-p-myristyroxycinnamic acid-1-monoglyceride
Chlorocyclizine	N-(Benzyloxy)-N-(3-phenylpropyl)acetamide-1-benzylimidazole
Aspirin	Acetyl salicyclic acid
TCE	Trichloroethylene
	Group C - Plasticizers and Related Compounds
DEHP	Di(2-ethylhexyl)phthalate
DEHA	Di(2-ethylhexyl)adipate
DEHS	Di(2-ethylhexyl)sebacate
	2-Ethylhexanol
	2-Ethyladehyde
	2-Ethylhexanoic acid
	2-(4-Dibenzofuranyloxy)-2-methylpropionic acid
	Group D - Dietary Manipulation
	High-fat diet
	Vitamin E deficiency diet

Sources: Reddy and Lalwani, 1983; Elcombe, 1985

Table 2. Nonmutagenic Peroxisome Proliferators That Have Induced Liver Tumors in Rodents [Ranked from highest (top) to lowest (bottom) in terms of capacity for peroxisome proliferation in rodents]

Methyl Clofenapate
Nafenopin
Tibric Acid
Wy-14,643
Clofibrate
Di(2-ethylhexyl)phthalate (DEHP)
BR-931
Ciprofibrate
Di(2-ethylhexyl)adipate (DEHA)
Bezafibrate

Sources: Reddy and Lalwani, 1983; Elcombe, 1985

carcinogens (e.g., aflatoxins, nitrosamines, and others). This research will evaluate the capacity of the fish model to respond to nongenotoxic carcinogens via the peroxisome proliferation mechanism. If it is shown that fish respond in a similar manner as rodents to peroxisome proliferators, then fish models could be employed as predictive tools to identify an important class of epigenetic carcinogens.

TECHNICAL OBJECTIVES

The research will involve testing seven peroxisome proliferators in the rainbow trout (*Salmo gairdneri*) and four peroxisome proliferators in both the goldfish (*Carassius auratus*) and Japanese medaka (*Oryzias latipes*). The agents to be tested were chosen because (1) they are the most potent peroxisome proliferators (Table 2), (2) they are carcinogenic, and (3) they are nonmutagenic in genotoxic assays. Dose-response and electron microscopy data will be collected for each agent administered to each fish species. In addition, a cancer bioassay will be conducted to evaluate whether peroxisome proliferation is associated with cancer induction in trout.

The objectives of this research are to characterize the ability of the fish model to develop peroxisome proliferation, to determine its comparability to the rodent response, and to ascertain the likelihood of cancer induction due to a peroxisome proliferator. The establishment of an extensive database on fish responses to peroxisome proliferators will indicate the relevance of the fish model for predictive testing of potential epigenetic carcinogens.

SIGNIFICANCE

The development of fish as an animal model for predictive testing of potential epigenetic carcinogens will allow for immediate testing of environmental pollutants or drugs that may have significant human health effects. Drugs or chemicals that induce cancer via a nongenotoxic mechanism cannot be detected in standard *in vitro* mutagenicity tests. A sensitive indicator of one class of epigenetic carcinogens may be the ability to induce peroxisome proliferation. If it is shown that fish are sensitive to known peroxisome proliferators and that they develop peroxisome proliferation and tumor production due to exposure to these agents, fish can then be used in place of rodents as animal models for this type of research.

The advantages of using fish as animal models rather than rodents include their small size, short generation time, reduced cost per experiment due to decreased space requirements for housing, and shorter latent periods for tumor induction compared to mammals. The development of fish as a sensitive animal model for testing of potential epigenetic carcinogens may provide a valid and more economical model for assessing the toxicity of chemicals that affect human health.

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DEVELOPMENT OF NEW CARCINOGENICITY BIOASSAYS

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INTRODUCTION

The purpose of this presentation is to summarize the current status of small fish carcinogenesis bioassays, especially the protocols and procedures used and developed at the Gulf Coast Research Laboratory, and to outline approaches for expanding the usefulness of the models and improving them. Small fish carcinogenesis bioassays have attracted widespread attention because of the many advantages and attributes they seem to offer for detecting and assessing environmental carcinogens, as well as for elucidating some basic mechanisms of carcinogenesis. Several small fish species that are being promoted as potential bioassay models are relatively inexpensive to rear, test, and maintain. They often respond rapidly to carcinogen exposure and facilitate histopathological analyses because whole specimens can be examined on a single glass slide. Furthermore, ambient water through which specimens can be exposed is an effective medium that insures uniform exposures.

EXPERIMENTAL MODELS

Attention at the Laboratory has been focused on two experimental models, the Japanese medaka (*Oryzias latipes*) and the king cobra strain of guppy (*Poecilia reticulata*). The egg-bearing medaka has several advantages for carcinogenesis bioassays over the live-bearing guppy. Large numbers (sometimes several thousand) of same-day-old medaka embryos can be obtained for testing. Medaka appear to be more genetically uniform than the guppy, and, in concurrent tests, medaka generally appear to be more sensitive to carcinogens than the guppy. The guppy, however, apparently has a lower spontaneous tumor rate than the medaka, is more resistant to the toxic effects of some carcinogens, and often develops different kinds of tumors than medaka when exposed to the same carcinogen.

EXPOSURE METHODOLOGY

Two exposure systems, static and flow-through, are utilized. Decisions on which system to use in testing a particular chemical depend on several factors, including: (1) the availability of the test chemical; (2) how much hazardous waste is to be generated; (3) the physicochemical

characteristics of the test chemical, such as its persistence in water and whether a solvent carrier is needed; and (4) whether the test compound is expected to be carcinogenic following brief exposures or whether prolonged exposures are probably necessary to test for carcinogenicity. Before carcinogenicity tests are conducted, the baseline toxicity of the compound in acute tests is estimated by range-finding tests and from the existing literature.

Static tests generally are conducted in a carcinogen glove box with 6- to 12-day-old specimens in 1-liter beakers. Depending on the toxicity and expected carcinogenic potency of the test compound, tests may range from a single 1- to 24-hour exposure to a series of exposures given on a weekly basis.

Chemicals chosen for flow-through tests generally have had very low water solubility and low expected carcinogenicity, or they were tested under those conditions to mimic some comparable situation such as long-term, low-level exposure of humans to carcinogens in drinking water. Exposures in this system begin with young specimens, are continuous, and have been as short as 28 days and as long as 6 months.

All tests, whether static or flow-through, incorporate the appropriate control groups including untreated (aquarium) controls, flow-through controls, and solvent (carrier) controls when necessary. Furthermore, analytical quantitation of test chemical concentrations in exposure media to determine actual exposure concentrations over the exposure periods is emphasized.

ENDPOINTS

Although factors such as mortality, growth, fecundity, and behavior are measured, tumorigenicity is the principal endpoint sought. Typically, samples for histopathological analysis are taken at 24 weeks and 36 weeks measured from the beginning of the exposure. About 75 to 100 specimens are taken at each sampling period. However, the Laboratory tries to retain maximum flexibility in both sampling periods and numbers of specimens sampled. For example, if no tumors are observed in a 24-week sample, the remaining specimens would then be apportioned over 36-week and 52-week samples. On the other hand, if substantial numbers of tumors are seen in a 24-week sample, a terminal 36-week sample could be taken. Whole fish specimens are usually processed for histopathology. By this method, most tissues and organs can be examined on a single glass slide. Some planned tests, however, will utilize exceedingly large numbers of specimens and will examine only liver tissues.

In the fish models, hepatic tissues appear to be the most sensitive to carcinogens. Three classes of hepatocellular neoplastic lesions (altered foci, adenomas, and carcinomas) have been related to carcinogen exposure. Cholangiocellular neoplasms (cholangiomas,

cholangiocarcinomas) tend to develop following exposure to toxic levels of carcinogens. Other carcinogen-induced neoplasms include: retinal neoplasms (medulloepithelioma, pigment epithelial carcinoma); exocrine pancreatic neoplasms (adenoma, carcinoma); neural neoplasms (neurilemmoma, ganglioma); soft tissue neoplasms (fibroma, fibrosarcoma, undifferentiated sarcomas, rhabdomyosarcoma); vascular neoplasms (histiocytoma, hemangioma, hemangiopericytoma); gallbladder carcinoma; kidney neoplasms (nephroblastoma, adenocarcinoma); and gill capillary hemangioma.

One attractive feature of fish in carcinogenesis models is their low rate of spontaneous neoplasms. Incidences for spontaneous hepatocellular lesions from control medaka tabulated to date are 1/635 (0.16%) at 24 weeks of age, 19/1448 (1.3%) at 36 weeks, and 5/250 (2.0%) at 52 weeks. In the guppy, incidences are 0/520 (0%) at 24 weeks, 5/975 (0.5%) at 36 weeks, and 5/347 (1.4%) at 52 weeks. Other spontaneous neoplasms occurring either in controls or in exposed specimens but unrelated to dose or compound include lymphosarcoma, germ cell neoplasms (seminoma, dysgerminoma), and swim-bladder carcinoma.

STUDIES UNDERWAY AND PLANNED

To expand the usefulness and improve the sensitivity of the medaka/guppy carcinogenesis model, carcinogenicity tests are being conducted on 2-acetylaminofluorene, cadmium, and tetrachloroethane, three of nine chemicals involved in current research efforts sponsored by the U.S. Army Medical Research and Development Command. Furthermore, to better understand the capabilities of small fish for metabolizing precarcinogens to their ultimate carcinogenic species, biochemical studies will be conducted on the ability of medaka to activate and detoxify 2-acetylaminofluorene (2-AAF) and to activate EDB as part of this study.

2-Acetylaminofluorene (2-AAF)

2-Acetylaminofluorene (2-acetamidofluorene; N-2-fluorenylacetamide) represents a class of chemicals, the aromatic amines, that includes several other well-known carcinogens such as aniline and benzidine. Although the carcinogenicity of 2-AAF in rodents is well known and it is widely used as a model carcinogen in initiation-promotion tests, its carcinogenicity, or the carcinogenicity of any other aromatic amine, has not been demonstrated in fish systems. The water solubility and persistence of 2-AAF and its acute toxicity to young medaka and guppies will be determined, and then carcinogenicity tests will be conducted in static and/or static-renewal systems. Exposure concentrations will be analyzed by flame-ionization gas-liquid chromatography at time zero and at the end of all tests exceeding 2 hours in length.

Approximately 300 guppies (2 days old) and medaka (6 to 7 days old) in each treatment group will be tested. Treatment groups will include: a control group, a solvent control group (if necessary), a low 2-AAF dose group, an intermediate 2-AAF dose group, and a high 2-AAF dose group. Intermittent or extended static renewal exposure may be employed. Samples for histopathology will be taken at 24, 36, and possibly 52 weeks post-exposure.

To determine how the medaka and guppy metabolize 2-AAF, N-hydroxylation, the process whereby the proximate carcinogen is produced, and ring hydroxylation, which is a detoxifying process, will be examined. For N-hydroxylation, liver homogenates will be incubated with radiolabeled 2-AAF and NADPH, and the resulting metabolite, N-hydroxy-2-AAF, will be assayed. To determine the fish's ability to produce the ultimate carcinogenic metabolite of 2-AAF, radiolabeled N-hydroxy-2-AAF will be incubated with fish liver cytosol, and the presence of the ultimate carcinogen, 2-AAF-N-sulfate, will be assayed. To determine detoxification abilities, the product of ring hydroxylation, 7-hydroxy-2-AAF, will be examined in liver homogenates, as well as the ability of hepatic microsomal enzymes to produce the glucuride of N-hydroxy-2-AAF in the presence of radiolabled N-hydroxy-2-AAF and VDP-glucuronide acid.

Cadmium

Cadmium (Cd) is a heavy metal contaminant of air, food, and water supplies. It induces malignant transformation in cultured cells, sarcomas at injection sites in rats, and lung tumors in rodents when administered as an aerosol. Cadmium has been correlated with a high incidence of prostatic and renal cancer in workers occupationally exposed to the metal.

The carcinogenicity of cadmium (Cd^{++} as $CdCl_2$) in medaka and guppies will be tested by exposing them to the metal through static and intermittent pulse-dose exposures in a single concentration, which will be approximately 80% of the lowest observable effect concentration (LOEC). The total cadmium dose will be varied by exposure length and number. Cadmium concentrations will be determined by atomic adsorption spectrophotometry immediately prior to addition of test fish and upon termination of each exposure.

1,1,2,2-Tetrachloroethane

Tetrachloroethane (TeCE) is a solvent used in cleaning processes and in the manufacture of paints, varnishes, and rust removers. It is used as a soil sterilizer, a weed killer, an insecticide, and in the manufacture of trichloroethylene and other chlorinated hydrocarbons. It has induced hepatocellular carcinoma in mice but not in rats. It will be of special interest to

compare this halogenated ethane to 1,2-dibromoethane, which is highly carcinogenic to medaka.

Medaka and guppies will be exposed to TeCE in the flow-through system for 90 days. Three exposure regimes will be utilized. Two of those will be intermittent 24-hour exposures, one near the LOEC and one about one-half the LOEC. Each 24-hour exposure to the fish will be followed by a period of 6 days in flowing, toxicant-free diluent water. This protocol will continue for 90 days. The third treatment will be a continuous and uninterrupted exposure for 90 days at about one-fourth the LOEC. Each treatment level will be quantitated by electron-capture gas chromatography from samples collected twice weekly.

VALIDATION OF THE MEDAKA ASSAY FOR CHEMICAL CARCINOGENS

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ABSTRACT

The objective of this project is to validate the use of the medaka carcinogenicity assay for identifying carcinogens among Agency priority chemicals. Current progress includes the following:

- Establishment of a standardized medaka carcinogenicity assay protocol for continuous aqueous exposures to chemicals.
- Selection of 48 carcinogens and 12 noncarcinogens for initial validation efforts.
- Compietion of medaka carcinogenicity assay exposures with 13 animal carcinogens and 2 noncarcinogens. Seven of these assays are in the "grow-out phase" of the assay.
- Pathology complete or in progress on medaka from four chemical exposures.

The three chemicals for which the pathology has been completed are: (1) hexachloro-benzene, which was negative; (2) aniline, which was positive, causing epithelioma of the gas bladder; and (3) 4-chloroaniline, also causing epithelioma of the gas bladder. Pathology examinations on di(2-ethylhexyl)phthalate (DEHP) are still in progress; however, preliminary results indicate that it is carcinogenic to medaka, causing cholangiocellular carcinoma.

INTRODUCTION

Chemically induced neoplasia is a multistep process that has common features in most vertebrate species studied thus far. The mechanisms of tumor induction in fish, rats, and mice have been shown to be similar, if not identical. There is, therefore, a comparative basis for considering fish carcinogenicity assays as having possible significance at a higher phyletic level. Fish carcinogenicity assays might be able to reliably predict the carcinogenic potential of chemicals for other species of fish and higher vertebrates.

While it is true that fish might predict animal carcinogens in general, much work is necessary before this predictive capacity can be established. Most fish carcinogenesis research has been oriented toward the carcinogenic process. Consequently, investigators have usually

chosen model chemicals that are relatively potent animal carcinogens, representing only a few carcinogenic mechanisms. The exposure methods used in these studies have varied greatly, making any comparisons difficult. Research is required to establish a validated fish carcinogenicity assay by testing a diversity of compounds with known activities and carcinogenic mechanisms as defined by rodent bioassays. In addition, the influence of exposure route and metabolism must be studied to provide potency estimates for each chemical based on the test organism and target organ. Molecular dose data would also be useful for sound risk assessment.

The research necessary to validate the fish carcinogenicity assay is, first, to establish a biologically sound protocol that would be expected to detect carcinogens not only with differing mechanisms, but also over a broad potency range. Then this assay should be tested on a selected group of chemicals with known carcinogenic activity.

The Environmental Research Laboratory (ERL)-Duluth has made substantial progress in the last year toward developing and validating a fish carcinogenicity assay. The medaka carcinogenicity assay protocol has been established. A committee of experts from the Environmental Protection Agency (EPA) and the National Institute of Environmental Health Sciences (NIEHS) selected 48 carcinogens and 12 noncarcinogens for initial validation research. Exposures based on the established protocol are complete for 15 of the selected chemicals. Pathology is complete on three chemicals--hexachlorobenzene, aniline, and 4-chloroaniline--and is in progress on a fourth chemical, DEHP.

CHEMICAL SELECTION

In September 1987, a meeting of the Ad Hoc Chemical Selection Committee was held to select chemicals for initial validation studies on the medaka carcinogenicity assay. Each member of the committee was given a list of 338 chemicals arranged in 29 different chemical classes. Chemicals on the list were the combined set of "sufficient positive" and "sufficient negative" carcinogens from the Gene-Tox database, and selected positive and negative carcinogens from the National Toxicology Program (NTP). The NTP list was derived from a recent study by NIEHS comparing *in vitro* and *in vivo* results. From this list, the committee selected 48 carcinogens (Table 1) and 12 noncarcinogens (Table 2), based on the following considerations:

- Carcinogens should be selected from several chemical classes.

Medaka Carcinogenicity Assay (9-30-87)

	CAS Number	Chemical
1	50000	Formaldehyde
2	50180	Cyclophosphamide
3	50293	p,p'-DDT
4	50555	Reserpine
5	51525	6-Propyl-2-thiouracil
6	51752	Nitrogen mustard
7	51796	Urethane
8	54886	2-Methyl-4-dimethylaminoazobenzene
9	55801	3'-Methyl-4-dimethylaminoazobenzene
10	56531	Diethylstilbestrol
11	57067	Allyl isothiocyanate
12	57147	1,1-Dimethylhydrazine
13	57578	beta-Propiolactone
14	67210	dl-Ethionine
15	71432	Benzene
16	78591	Isophorone
17	91598	2-Naphthylamine
18	95807	2,4-Diaminotoluene
19	96457	N,N'-Ethylenethiourea
20	100754	N-Nitrosopiperidine
21	106934	Ethylene dibromide(1,2-Dibromoethane)
22	107073	2-Chloroethanol
23	117817	Di(2-ethylhexyl)phthalate (DEHP)
24	118741	Hexachlorobenzene
25	122667	Hydrazobenzene
26	123911	1,4-Dioxane
27	126727	Tris(2,3-dibromopropyl)phosphate
28	134292	o-Anisidine hydrochloride
29	140114	Benzyl acetate
30	140885	Ethyl acrylate
31	150685	Monuron
32	154938	Bis-N-N'-(chloroethyl)-nitrosourea
33	512561	Trimethyl phosphate
34	531851	Benzidine hydrochloride
35	540738	1,2-Dimethylhydrazine
36	542756	1,3-Dichloropropene
37	602879	5-Nitroacenaphthene
38	759739	N-Nitroso-N-ethylurea
39	1746016	2,3,7,8-TCDD
40	3771195	Nafenopin
41	5131602	4-Chloro-m-phenylenediamine
42	7227910	1-Phenyl-3,3-dimethyltriazene
43	7488564	Selenium sulfide
44	7789062	Strontium chromate
45	10034932	Hydrazine sulfate
46	12035722	Nickel subsulfide
47	24554265	N-[4-(5-Nitro-2-furyl)-2-thiazolyl] formamide
48	26471625	2,4 & 2,6-Toluene diisocyanate mix

Table 2. Noncarcinogenic Chemicals Chosen for Validation of the Medaka Carcinogenicity Assay (9-30-87)

	CAS Number	Chemical
1	72435	Methoxychlor
2	77656	Carbomal
3	91203	Napththalene
4	95501	1,2-Dichlorobenzene
5	108952	Phenol
6	109693	Chlorobutane
7	116063	Aldicarb
8	134327	1-Naphthylamine
9	148243	8-Hydroxyquinoline
10	333415	Diazinon
11	624180	p-Phenylenediamine dihydrochloride
12	2481949	N,N-Diethylaminoazobenzene

- Noncarcinogens should be selected from the same classes as the carcinogens, if possible.
- A range of carcinogenic potency should be represented.
- Chemicals with different carcinogenic mechanisms should be selected.

From this list of 48 carcinogens and 12 noncarcinogens, an EPA Office of Toxic Substances (OTS) committee selected 21 chemicals (Table 3) for immediate testing in the validation effort. In the first quarter of FY88, ERL-Duluth began the first exposures to carcinogens from the OTS list of chemicals.

Prior to the meeting of the Ad Hoc Chemical Selection Committee and the OTS committee, ERL-Duluth had already started or completed medaka exposures with aniline, 4-chloroaniline, and hexachlorobenzene. Thus, even though aniline and 4-chloroaniline were not included on the chemicals selected by the Ad Hoc Committee (Tables 1 and 2) and hexachlorobenzene was not included by the OTS committee (Table 3), results from exposures to these chemicals are in this report.

MEDAKA CARCINOGENESIS ASSAY PROTOCOL

Biology

If a chemical is relatively water soluble, a 28-day exposure protocol is followed. Fish are exposed, using a serial flow-through diluter, to five different concentrations and a control.

Table 3. Chemicals Selected by EPA, OTS/HERD for Initial Testing in the Medaka Carcinogenicity Assay

	Case Number	Chemical
1	50000	Formaldehyde
2	50293	p,p' DDT
3	51796	Urethane
4	55801	3'-Methyl-4-dimethylaminoazobenzene
5	57067	Allyl isothiocyanate
6	57147	1,1-Dimethylhydrazine
7	67210	dl-Ethionine
8	91598	2-Naphthylamine
9	94757	2,4-Dichlorophenoxyacetic acid (2,4-D)
10	95807	2,4-Diaminotoluene
11	100754	N-Nitrosopiperidine
12	106934	1,2-Dibromoethane (Ethylene dibromide)
13	116063	Aldicarb
14	117817	Di(2-ethylhexyl)phthalate (DEHP)
15	123911	1,4-Dioxane
16	140885	Ethyl acrylate
17	531851	Benzidine hydrochloride
18	540738	1,2-Dimethylhydrazine
19	542756	1,3-Dichloropropene
20	2481949	N,N-Diethylaminoazobenzene (N,N-DEAAB)
21	3771195	Nafenopin

The highest concentration used is the 96-hour LC_{50} value derived from a preliminary acute test. The typical serial dilution factor is 0.5, resulting in exposure concentrations of 1.0, 0.5, 0.25, 0.125, and 0.063 of the 96-hour LC_{50}. These five concentrations and the control are duplicated. Sixty 1- to 4-day post-hatch medaka are added to each of the twelve 2-liter exposure tanks. The test is maintained at 25°C, with flow rates to each tank of 25 ml/min and a photoperiod of 14 light/10 dark. The fish are fed freshly hatched brine shrimp twice daily (once daily on weekends). These conditions are maintained for 28 days. At the end of the exposure, survival and fish weight are recorded as a measure of toxicity. The fish are then transferred into clean water.

Chemicals that are nontoxic at aqueous saturation in a typical 28-day exposure are exposed using a different protocol. Usually these are chemicals with a high log P and low water solubility. These tests use only one exposure concentration (aqueous saturation) and a control, both run in duplicate. Two hundred 1- to 4-day post-hatch medaka are added to each of the four 15-liter exposure tanks. The tanks are maintained at 25°C with flow rates of

100 ml/min and a photoperiod of 14 light/10 dark. The fish are fed freshly hatched brine shrimp twice daily (once daily on weekends). These conditions are maintained for up to 6 months. However, as a reference to the standard 28-day exposure, 60 fish are randomly selected and moved to grow-out at 28 days. The remaining fish stay in exposure for an additional 5 months unless mortality begins to occur. If mortality begins to occur, then the exposure is terminated and the fish are transferred to clean water. During this type of exposure, fish are subsampled at appropriate times for tissue residue analysis and histological assessment.

When an exposure is successfully completed, the fish are weighed and moved into grow-out tanks that receive clean flow-through dilution water. The temperature is maintained at 25°C with flow rates of 100 ml/min and a photoperiod of 8 light/16 dark. The fish are fed freshly hatched brine shrimp twice daily (once daily on weekends). Observations are made on a daily basis when the fish are in grow-out. Moribund fish are removed and fixed to insure that they can be analyzed. The fish are held in grow-out for 22 weeks.

At the end of the grow-out period, fish are anesthetized using tricaine methanesulfonate, assigned a specimen number, weighed, examined for gross lesions, and fixed in 15 ml of HEPES-buffered 10% formaldehyde (pH 7.4). The vials containing the fish and fixative are placed on a shaker table and gently agitated overnight to facilitate fixation. They are then placed in cold storage (4°C) for at least 1 week to complete fixation. When fixation is completed, the fish are dehydrated through a graded series of ethanol and embedded in a water-soluble glycol methacrylate plastic. The embedded specimens are cut on a microtome at 3 μm. Sagittal and parasagittal selections are mounted on glass slides and stained with hematoxylin and eosin. This orientation of the section ensures that all of the organs of interest are contained on at least one of three slides which are prepared for analysis. Tissues are analyzed on a light microscope, and observations are recorded on a data sheet in a coded format. This information is then entered into computer files for further analysis.

Histological analysis is done on all dead or moribund fish observed in grow-out as well as on fish sampled from each concentration at the end of the 28-day exposure. The exposure concentration, which is defined as the maximum tolerated concentration (MTC), is the exposure concentration with some, but not more than 10% mortality, in the 28-day exposure. This group and any survivors from higher exposure concentrations are analyzed first for the presence of neoplasms or preneoplastic foci. If tumors are present in these groups, the controls are analyzed. The fish exposed to the 0.5 dilution of the MTC are embedded and may be analyzed. Sometimes the mortality dose-response curve is very steep, resulting in 100%

mortality in one concentration and no mortality in the adjacent lower concentration. In this case, the MTC is the highest concentration with surviving fish.

Chemistry

The chemical used in each test is the highest purity available that is not prohibitively expensive (i.e., not greater than $4,000 to run the test). Usually this results in greater than 99% purity. However, chemicals are never used if they are less than 95% pure. In all cases, the nature of the impurities is considered before deciding to start a test.

Depending on the physical characteristics of a particular chemical, one of several techniques is used to prepare stock chemical solutions. These include: direct solution, high-speed mixing, liquid/liquid saturation, and column saturation. The stock solutions are pumped into the exposure apparatus for dilution and delivery to the exposure tanks.

The appropriate analytical techniques are developed prior to the start of an exposure. Typically, high-performance liquid chromatography, gas chromatography, and gas chromatography/mass spectrometry are used to analyze and verify the aqueous concentration of a test chemical. This analysis is conducted at least weekly for each tank in the diluter.

Tissue residue levels are also measured for tests of relatively insoluble chemicals that are nontoxic at saturation in a typical 28-day test. This analysis is used to document the bioaccumulation of the chemical by the organism and to establish a tissue residue level to correlate with either toxicity or carcinogenicity. This measurement is important because the aqueous concentrations for these chemicals are not useful to correlate with observed effects.

STATUS OF SELECTED TEST CHEMICALS

Exposures

Medaka exposures are complete on 15 test chemicals, and the status of each test is shown in Table 4. The measured chemical concentrations of these exposures and the percent survival after 28 days of exposure are indicated in Table 5. Of these 15, hexachlorobenzene and DEHP were exposed according to the initial 28-day exposure protocol as well as to modified exposure protocols for low-solubility chemicals (Table 4). Ten additional chemicals are being investigated for potential exposures. Several of these chemicals are too costly for testing at this time. N-nitrosopiperidine, for example, a high-priority chemical for validation, must be synthesized for this project. Costs are estimated at $8,000 for this synthesis. dl-Ethionine, which is nontoxic to medaka at high aqueous concentrations, is also too expensive. Currently, exposures are continuing at a rate of about 1 per month.

Table 4. August 1988 Status of Chemicals Selected for Testing

CAS #	Chemical Name	Exposure Planned	In Exposure	In Grow-Out	In Histology	In Analysis
62533	Aniline*					+
106478	4-Chloroaniline*					+
118741	Hexachlorobenzene*					+
50000	Formaldehyde				+	
57147	1,1-Dimethylhydrazine				+	
107073	2-Chloroethanol				+	
108952	Phenol				+	
117817	DEHP (28 day)				+	
140885	Ethyl acrylate				+	
51796	Urethane			+		
94757	2,4-D			+		
95807	2,4-Diaminotoluene			+		
106934	1,2-Dibromoethane			+		
117817	DEHP (6 month)			+		
123911	1,4-Dioxane			+		
542756	1,3-Dichloropropene			+		
50293	p,p'-DDT	+				
55801	3'-Methyl-4-DNAAB	+				
57067	Allyl isothiocyanate	+				
62710	dl-Ethionine	+				
91598	2-Naphthylamine	+				
100754	N-Nitrosopiperidine	+				
116063	Aldicarb	+				
1746016	2,3,7,8-TCDD	+				
2481949	N,N-DEAAB	+				
3771195	Nafenopin	+				

*Exposure began before chemical selection meetings; thus, not shown on all selection lists (Tables 1, 2, and 3).

Table 5. Aqueous concentration (mg/l unless noted) measured in each tank and % survival (in parentheses) at the end of exposure.

CAS #	Chemical Name	Tank	1		2		3		4		5		6		Methods	Detection limits
		Replicate	A	B	A	B	A	B	A	B	A	B	A	B		
50000	formaldehyde		0 02 (95)	0 02 (100)	2 57 (95)	2 26 (98)	5 86 (100)	5 19 (100)	10 60 (93)	11 00 (93)	21 90 (95)	23 80 (95)	48 60 (95)	47 60 (87)	HPLC DNPH Der	0 01 mg/l
51796	urethane	<	4 00 (100)	4 00 (97)	412 (97)	428 (97)	559 (97)	545 (97)	933 (100)	908 (95)	1870 (85)	1910 (73)	3860 (0)	4040 (0)	GC Aq Inj	4 00 mg/l
57147	1,1-dimethylhydrazine	<	0 10 (90)	0 10 (98)	1 51 (100)	1 44 (98)	2 56 (95)	2 32 (95)	4 07 (100)	4 27 (98)	8 31 (45)	8 17 (42)	16 90 (0)	17 30 (0)	HPLC Ac Der	0 10 mg/l
62533	aniline	<	1 00 (98)	1 00 (97)	4 77 (93)	4 57 (90)	8 81 (80)	8 88 (82)	18 20 (60)	18 20 (73)	37 30 (53)	37 70 (45)	74 40 (42)	74 20 (15)	GC Aq Inj	1 00 mg/l
94757	2,4-D	<	0 80 (97)	0 80 (98)	25 50 (97)	29 00 (92)	55 80 (87)	55 40 (85)	110 00 (40)	114 00 (48)	217 00 (0)	222 00 (0)	422 00 (0)	422 00 (0)	HPLC Aq Inj	0 80 mg/l
95807	2,4-diaminotoluene	<	0 70 (97)	0 70 (100)	154 (98)	159 (95)	296 (67)	281 (63)	547 (5)	548 (8)	1061 (0)	1052 (0)	2087 (0)	1984 (0)	GC Aq Inj	0 70 mg/l
106478	4-chloroaniline	<	0 50 (97)	0 50 (98)	2 40 (98)	2 28 (93)	4 34 (98)	4 29 (100)	8 55 (95)	8 59 (93)	16 30 (83)	16 80 (83)	32 00 (32)	32 80 (37)	GC Aq Inj	0 50 mg/l
106934	1,2-dibromoethane	<	1 00 (100)	1 00 (98)	1 45 (100)	1 46 (100)	2 45 (100)	2 40 (95)	5 55 (100)	5 76 (100)	9 43 (100)	9 15 (10)	19 70 (32)	19 20 (35)	GC Hex Ext	1 00 mg/l
107073	2-chloroethanol	<	0 30 (100)	0 30 (98)	1 98 (95)	2 21 (100)	4 00 (100)	4 57 (100)	8 31 (97)	8 87 (100)	17 00 (73)	17 70 (82)	32 00 (45)	33 50 (47)	GC Aq Inj	0 30 mg/l
108952	phenol	<	0 05 (97)	0 05 (100)	0 95 (100)	1 41 (98)	2 31 (100)	2 95 (98)	5 45 (93)	6 34 (98)	12 00 (98)	12 20 (98)	30 30 (0)	29 20 (0)	HPLC Aq Inj	0 05 mg/l
117817	DEHP (28 day)		0 09 (99)	0 08 (98)	0 54 (97)	0 57 (99)	--	--	--	--	--	--	--	--	GC Hex Ext	0 03 mg/l
117817	DEHP (6 month) (nominal conc)		0 00 (99)	0 00 (95)	0 40 (89)	0 40 (93)	--	--	--	--	--	--	--	--	-- ---	-- mg/l
118741	hexachlorobenzene (μg/l)		0 01 (93)	--	5 20 (91)	--	--	--	--	--	--	--	--	--	GC Hex Ext	0 01 ug/l
123911	1,4-dioxane		<50 00 (93)	<50 00 (97)	547 (97)	582 (98)	978 (100)	1010 (100)	1830 (95)	1910 (93)	3550 (88)	3520 (95)	6900 (83)	6960 (85)	GC Aq Inj	50 00 mg/l
140885	ethyl acrylate	<	0 07 (88)	0 07 (97)	0 18 (95)	0 19 (100)	0 32 (98)	0 29 (100)	0 52 (95)	0 50 (100)	1 04 (33)	1 02 (28)	2 10 (0)	2 01 (2)	GC Aq Inj	0 07 mg/l
542756	1,3-dichloropropene	<	0 01 (100)	0 01 (100)	0 12 (100)	0 13 (92)	0 24 (97)	0 26 (97)	0 50 (97)	0 51 (98)	1 11 (75)	1 27 (75)	2 56 (0)	2 66 (0)	GC Hex Ext	0 01 mg/l

KEY TO ABBREVIATIONS

GC = Gas chromatography
HPLC = High performance liquid chromatography
Aq Inj = Direct aqueous injection
Hex Ext = Hexane extraction
Ac Der = Acetone derivitization
DNPH Der = Dinitrophenylhydrazine derivitization

4-Chloroaniline Results

Seventy-six fish exposed to 16.5 mg 4-chloroaniline/liter were analyzed and compared to 100 control fish from the same cohort. Two pathological lesions were consistently observed in the exposed group. The first, a tumor of the gas bladder secretory epithelium, occurred in 13 fish or 17% of the group. This tumor has not been described in the literature. Consequently, its characteristics are unknown. However, this tumor often nearly filled the gas bladder lumen, causing the affected fish to lose buoyancy control. This tumor was marked by the following characteristics: pleiomorphic nuclei, high mitototic activity, polynucleate cells, and cellular hypertrophy (Figure 1). It was well organized and well vascularized. However, it was never noted outside of the confines of the gas bladder.

The second lesion, which was also seen in 17% of the exposed fish, was a multifocal cystic proliferation of bile duct tissue in the liver (Figure 2). This cystic hyperplasia displaced varying amounts of the liver parenchyma, but was not invasive. This lesion is probably not neoplastic because the morphology of the cells is consistently normal and well differentiated and their mitotic activity is relatively low.

One additional lesion, a teratoma of the ovary, was diagnosed in one of the exposed fish that also had the multifocal cystic hyperplasia of bile duct in the liver. This unusual growth included well-differentiated brain tissue, cartilage, renal tubules, ova, and several other unidentified tissues within the tunica of the ovary (Figure 3). Because this is a single occurrence, it is unclear if this lesion is chemically induced. However, this type of lesion is very rare in fish.

The 100 control fish did not have any of the above-mentioned lesions, but one tumor (1% of the group), tentatively identified as a leiomyosarcoma, was observed. This lesion was distributed widely throughout the viscera of one fish and was involved with much of the gut and ovary.

Aniline Results

Nineteen medaka exposed to the high concentration of aniline (74.3 mg/liter) survived until the end of grow-out and were examined for neoplastic lesions. In the next lower concentration (37.5 mg/liter) 31 fish survived for final analysis. In the control tanks, 99 fish survived, of which 67 were analyzed histologically for lesions. Only one lesion was consistently observed in the aniline-exposed fish, a tumor of the gas bladder secretory epithelium. This lesion has not been described in the literature; however, it is the identical lesion observed in medaka exposed to 4-chloroaniline. While the tumor had high mitotic

Figure 1. (A) Photomicrograph of the gas bladder of a control medaka demonstrating the normal thickness of the secretory epithelium (45x). (B) Photomicrograph of a gas bladder tumor in a medaka exposed to 4-chloroaniline for 28 days and sampled after 22 weeks of grow-out. Note that the tumor fills the lumen of the gas bladder (45x).

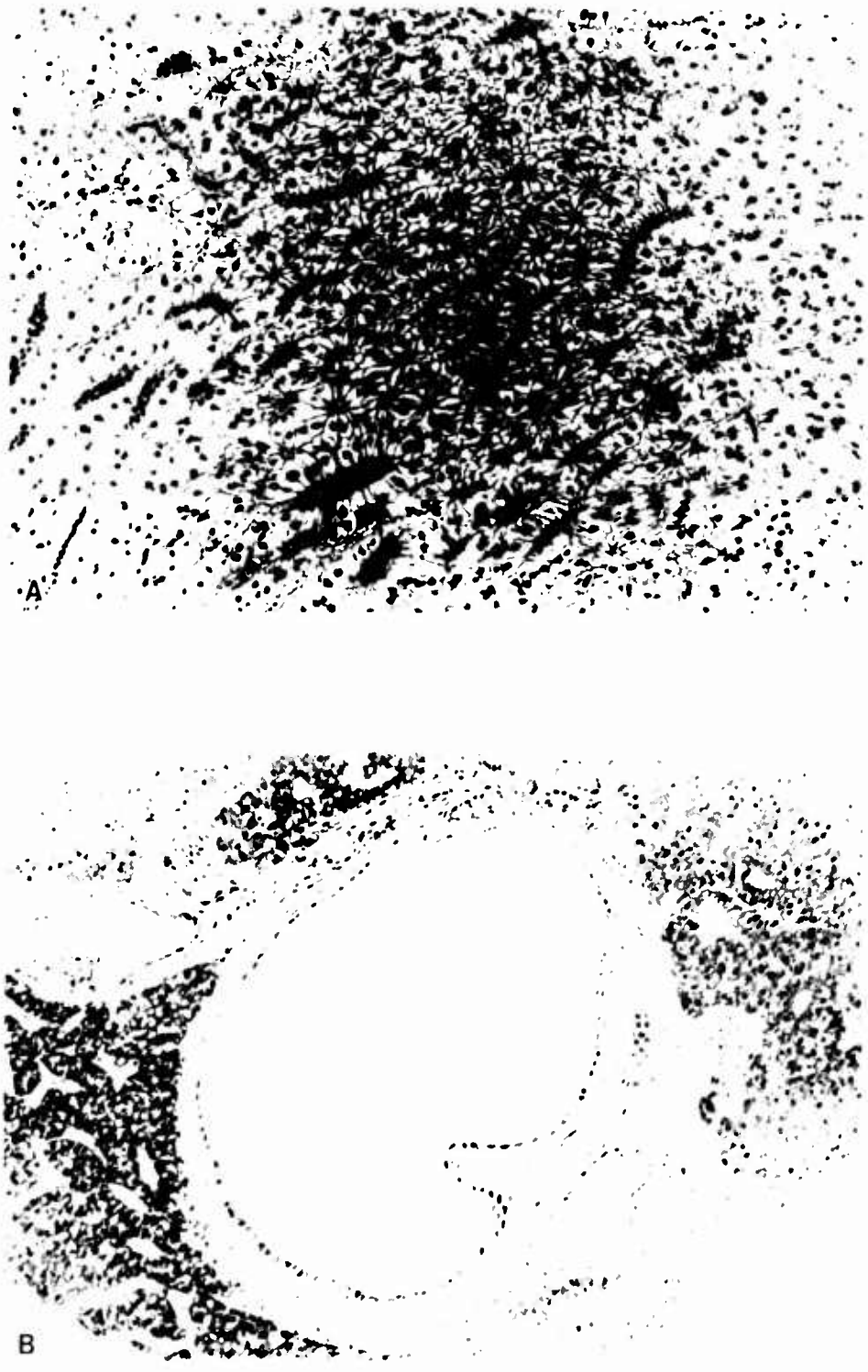

Figure 2. (A) Photomicrograph of a normal liver from a control medaka (225x). (B) Photomicrograph of a liver from a medaka exposed for 28 days to 4-chloroaniline and sampled at the end of 22 weeks of grow-out. The large cystic lesions are hyperplastic epithelial cells of the biliary system (225x).

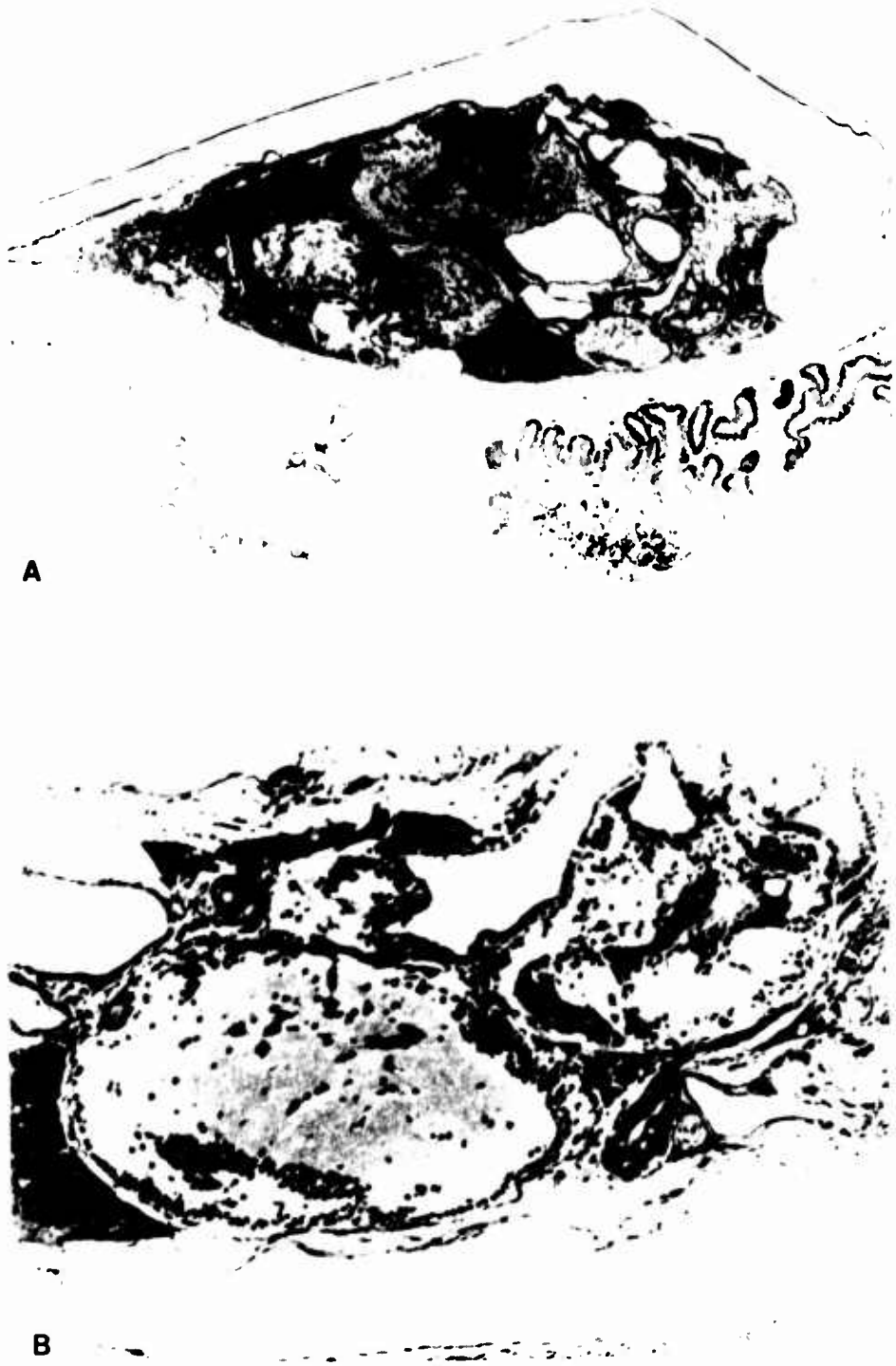

Figure 3. (A) Photomicrograph of a teratoma in the ovary of a medaka exposed to 4-chloroaniline for 28 days and sampled at the end of 22 weeks of grow-out. This lesion contains neural, renal, biliary, cartilagenous, and ovarian tissues (45x). (B) Higher magnification of the same teratoma in (A) demonstrating the various tissue types (225x).

activity, was well vascularized, and often nearly filled the lumen of the gas bladder, it never was observed to be invasive.

Seven out of 19 (36.7%) fish from the high exposure (74.3 mg aniline/liter) protocol had the gas bladder tumor, while 21 of the 31 (67.7%) fish from the lower concentration (37.5 mg aniline/liter) tank had this lesion. No lesions were observed in the control fish. The higher incidence observed in the lower concentration exposures is explained by high mortality in the higher exposure concentration, perhaps due to the tumor. This is difficult to confirm because dead fish were difficult to diagnose due to tissue degeneration. However, the tumor frequency from sampled moribund fish was very high.

DEHP Results

The modified exposure protocol was used for testing DEHP because a saturated solution (approximately 400 μg/liter) is nontoxic to medaka. This exposure began by placing 200 medaka larvae into each of four tanks, two DEHP tanks and two control tanks. After 28 days, 60 fish were transferred from each tank into the grow-out facility. Others were sampled at 1 month, 2 months, 3 months, and 6 months of continuous exposure for tissue residue analysis and for cytological observations using the electron microscope. Five and one-half months into the exposure, increased mortality was observed in the DEHP-exposed group. At 6 months, all remaining fish were transferred to grow-out. However, 6 males and 6 females were sampled from each tank for pre-grow-out histological analysis. Pathology studies on these 12 fish revealed a cholangiolar carcinoma in one specimen (Figure 4). The electron microscope analysis indicated that a peroxisome-like organelle may be proliferating in the livers of DEHP-exposed medaka. However, neither quantification nor definitive cytochemistry are complete at this time. The balance (approximately 60 in each replicate) of the DEHP-exposed fish are in the grow-out unit and will be analyzed in the future for tumor development.

Hexachlorobenzene Results

Hexachlorobenzene exposures were initiated before the protocols previously described were established. Consequently, the hexachlorobenzene exposure is slightly different. Medaka were exposed to a saturated solution (5.2 μg/liter) of hexachlorobenzene and to control water only. Exposure tanks were not duplicated. Fish were transferred to grow-out in two groups, after 28 and 56 days of exposure. All the fish were sacrificed for histological

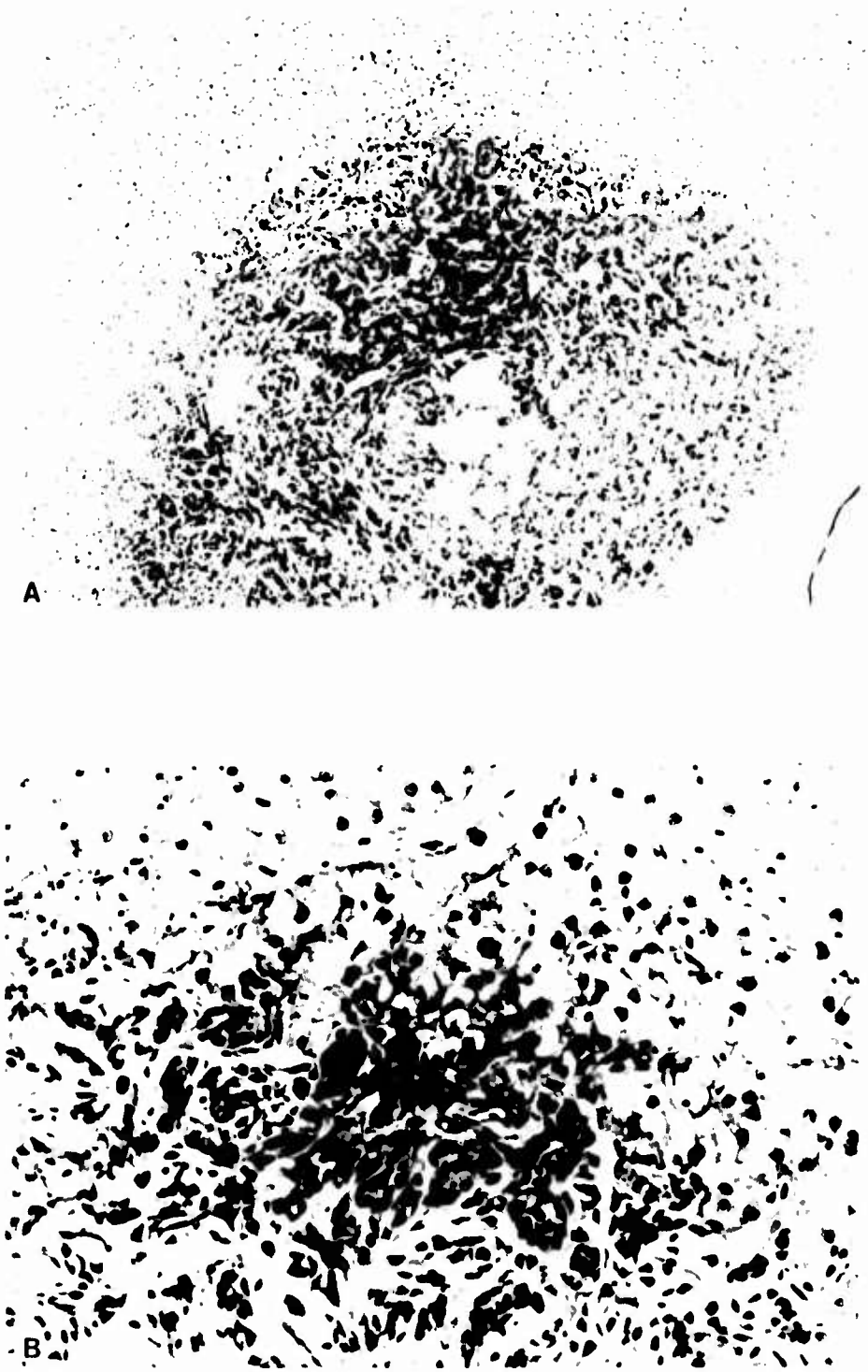

Figure 4. (A) Photomicrograph of a cholangiocellular carcinoma in the liver of a medaka exposed to DEHP for 6 months and sampled at the end of the exposure with no time in grow-out (110x). (B) Higher magnification of the tumor showing invasion of the tumor into normal liver parenchyma (450x).

analysis when they were 6 months old. Observations were made on 47 of the 28-day exposed fish and on 54 controls. No neoplastic lesions were observed in any of the fish.

CONCLUSIONS

Few assays are complete through histological analyses, but the results obtained thus far are promising. The induction of tumors by two different primary aromatic amines suggests that medaka are sensitive to this chemical class. More tests with both carcinogenic and noncarcinogenic primary aromatic amines will further clarify the sensitivity and selectivity of the assay. The preliminary results with DEHP indicate assay sensitivity to peroxisome-proliferating chemicals as well. More tests on this class will be very useful.

At this time, it is difficult to ascertain whether the protocol needs modification. However, the protocol modification for low-solubility, nontoxic chemicals seems appropriate. Research in this area is necessary to define the chemical characteristics that obligate using this modified protocol. Other protocol modifications to be considered include increasing the duration of the grow-out period and starting with older fish, which are usually more tolerant of the chemical, resulting in a higher MTC.

NEOPLASIA IN FISH: TUMOR AND MECHANISM STUDIES IN TROUT

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INTRODUCTION

The long-range goals of this project are to understand the significance to human health of epizootics of neoplasia in feral fish populations and to expand basic knowledge of the comparative aspects of neoplasia in fish and mammals. Although suspect etiologic agents such as benzo(a)pyrene (BP) and carbazole (CBZ) have been identified in some epizootic locales, causal relationships have not been established. Definitive associations will require not only direct demonstration of carcinogenic potencies in fish but also an understanding of the impact on tumor response of confounding variables including: water quality and temperature, nutritional status and growth rate, life stage at risk, genetic variation, population age structure, migration habits, and presence of tumor modulators as well as genotoxins. Further lacking is an understanding of the basic molecular biology of carcinogenesis in fish compared to mammals.

Although rainbow trout (*Salmo gairdneri*) seldom inhabit polluted environments where fish tumor epizootics occur, they have received over 20 years of study as a tumor model and thus are among the few laboratory fish tumor models with sufficient development to address many of these needs. It is proposed to use this model with the following specific aims: (1) conduct tumor studies to establish or confirm the carcinogenicities of BP, CBZ, and the model carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) in trout by selected exposure routes, and determine the impact on tumor response of potential modulating variables including temperature, nutritional status, selected dietary cofactors (DDT, CBZ, phthalates, Aroclor 1254), and maternally accumulated embryonic Aroclor 1254; (2) conduct mechanism studies on procarcinogen pharmacokinetics, cellular metabolism, DNA adduct formation and repair, and the influence of the above modulating influences on these processes; (3) further characterize fundamental aspects of tumor progression in trout, including development and progression of preneoplastic foci in liver and the transplantability of various tumor types into syngeneic hosts; and (4) investigate the possible involvement of oncogenes and tumor-transforming genes in trout neoplasia, through the isolation and characterization of selected c-*onc* genes,

determination of their expression and multiplicity in normal and tumor tissue, and development of cell culture systems for oncogenic transfection assay.

During the first 1½ years of the project, considerable progress has been made on each of these specific aims. That progress will be summarized by specific aim in the following paragraphs.

SPECIFIC AIM 1

The first objective of this aim was to establish appropriate exposure routes and doses of selected polycyclic aromatic hydrocarbons that would give predictable incidences of liver or other organ neoplasms in rainbow trout. Equipped with this knowledge, the proposed tumor modulation studies could then be conducted. Earlier, the carcinogenicity of BP in a fish species had been established by both dietary and intraperitoneal injection routes of exposure (Hendricks et al., 1985). Neither of these exposure routes, however, was amenable to the planned experiments due to the long latency periods involved. The next exposure route tested was embryo microinjection (Black et al., 1985) of BP. Surprisingly, over a dose range of 0.5 to 4 μg/embryo, mortalities ranged from 46 to 97%, while liver tumor incidence was only 2 to 7%. Mortalities occurred about 3 weeks after injection, near the end of yolk-sac absorption. This may have resulted from the mobilization of most of the dose, which was stored in yolk lipid, into a toxic surge of BP. An 8-μg/embryo dose of BP caused 100% mortality. Thus BP appeared to be unacceptable. Next, DMBA was microinjected into rainbow trout embryos at doses of 0.13, 0.84, 3.2, and 16.0 μg/embryo. Liver tumor incidences were approximately 5, 7, 31, and 15%, respectively, but mortalities were again high, reaching 84 and 97% at the two higher doses. Thus, although the tumor response was higher than with BP, the mortalities with DMBA were unacceptably high.

Based on a higher solubility of DMBA than BP, the static exposure of both embryos and fry to various doses of DMBA for variable periods of time was attempted with both single and multiple exposures. These experiments, presented in Tables 1 and 2, were highly successful, producing high incidences of both liver and stomach neoplasms and lower incidences of kidney and swim-bladder tumors, in addition to providing several combinations of dose, time, and number of exposures which will be useful in subsequent experiments on modulation. Thus, static exposure of either embryos or fry to aqueous solutions of DMBA provides a convenient model system with which to study the effects of various environmental conditions on the outcome of neoplasia in rainbow trout.

Table 1. Gross Tumor Incidence at 9 Months in Rainbow Trout Exposed to DMBA As Embryos

DMBA Embryo Exposure	Tumor Incidence					
	Liver	%	Stomach	%	Kidney	%
1 ppm/2 hr	3/130	2	0/130	0	0/130	0
5 ppm/2 hr	19/123	15	1/123	1	0/123	0
5 ppm/24 hr	99/125	79	15/125	12	4/125	3.2
1% DMSO/24 hr	0/118	0	0/118	0	0/118	0

Table 2. Gross Tumor Incidence at 9 Months in Rainbow Trout Exposed to DMBA as Fry

Fry Exposure	Tumor Incidence							
	Liver	%	Stomach	%	Kidney	%	Swim Bladder	%
1 ppm DMBA/1 hr	0/121	0	10/121	8	0/121	0	0/121	0
1 ppm DMBA/5 hr	9/122	7	56/122	46	0/122	0	1/122	1
1 ppm DMBA/5 hr 3 exp.--weekly	50/124	40	117/124	94	2/124	2	2/124	2
1 ppm DMBA/20 hr	42/125	34	95/125	76	1/125	1	1/125	1
1 ppm DMBA/20 hr 3 exp.--weekly	52/118	44	112/118	95	0/118	0	6/118	5
5 ppm DMBA/1 hr	37/120	31	109/120	91	0/120	0	6/120	5
5 ppm DMBA/5 hr	49/128	38	120/128	94	1/128	1	8/128	6
5 ppm DMBA/5 hr 3 exp.--weekly	20/38	53	36/38	95	1/38	3	7/38	18
5 ppm DMBA/20 hr	8/100	81	97/100	97	0/100	0	12/100	12
5 ppm DMBA/20 hr 3 exp.--weekly	4/21	19	9/21	43	1/21	5	0/21	0
1% DMSO/20 hr 3 exp.--weekly	0/122	0	0/122	0	0/122	0	0/122	0

Using both embryo and fry exposures to DMBA, a large experiment was initiated this year to determine the effects of selected environmental contaminants, such as diethylhexylphthalate (DEHP), DDT, Aroclor 1254 (PCB), and carbazole (CBZ), on the carcinogenicity of DMBA (Table 3). Dietary exposure to these compounds prior to fry initiation by DMBA and dietary exposure after embryo initiation by DMBA were used to investigate both inhibitory and/or enhancing effects, respectively. These experiments are scheduled for termination in September and November 1988.

SPECIFIC AIM 2

The metabolism of BP by rainbow trout *in vivo* and *in vitro* was studied to determine the activation and detoxication pathways of BP in the liver of trout, which is a target organ of BP-induced carcinogenesis. Trout were exposed to [^{3}H]BP intraperitoneally at dosages of 2 and 25 mg/kg fish. After 24 hours and 72 hours, the types of conjugated BP metabolites present in bile were determined by ion-pair high-performance liquid chromatography (HPLC). Biliary metabolites present in trout consisted mainly of the glucuronic acid conjugates of BP-7,8-dihydrodiol, 1-hydroxyBP, and 3-hydroxyBP at both dosages of BP. A single, major thioether conjugate arising from an unknown BP metabolite was detected in bile by ion-pair HPLC. The proportion of this metabolite in bile appears to be dosage dependent (7.6±0.9% at the lower dosage, 13±1% at the higher dosage).

No sulfate conjugates of BP metabolites were observed at either dosage of BP given to trout. The presence of only a single apparent thioether conjugate in bile of BP-exposed trout indicates that detoxication of electrophilic metabolites may not be an efficient process in trout liver. Binding of BP metabolites to hepatic DNA increased with time from 24 to 72 hours after BP exposure, suggesting that the rate of DNA repair was low and that continuous formation of BP-DNA adducts occurs over time. Microsomal metabolism of BP showed that the major metabolites formed included the BP-9,10-dihydrodiol, BP-7,8-dihydrodiol, BP quinones, 9-hydroxyBP, 1-hydroxyBP, and 3-hydroxyBP. No BP-4,5-dihydrodiol was detected.

Two major DNA adducts were observed *in vitro*: one was identified as the (+)-anti-BPDE-deoxyguanosine and the other had an identical retention time as the 9-hydroxyBP-4,5-oxide-deoxyguanosine, the major adduct formed by control Sprague Dawley rat microsomes. However, because K-region metabolism of BP was not otherwise detected in trout liver, the identity of this second adduct is uncertain. These results indicate that rainbow trout liver has the capability to metabolize BP into the ultimate carcinogenic form, anti-BPDE, and that

Table 3. Protocol for Modulation of DMBA Carcinogenesis in Trout by Dietary Aroclor 1254 (PCB), DDT, DEHP, or CBZ

Treatment Group (in duplicate)[1]	Embryo	Treatment During Development[2] Weeks 0-8 Post-hatch	Weeks 5-8 Post-hatch	Weeks 9 to Termination
1	-	DEHP (2,500 ppm)	+ DMBA	OTD[3]
2	-	DEHP (5,000 ppm)	+ DMBA	OTD
3	-	PCB (100 ppm)	+ DMBA	OTD
4	-	PCB (500 ppm)	+ DMBA	OTD
5	-	DDT (25 ppm)	+ DMBA	OTD
6	-	DDT (50 ppm)	+ DMBA	OTD
7	-	CBZ (500 ppm)	+ DMBA	OTD
8	-	CBZ (1,000 ppm)	+ DMBA	OTD
9	-	OTD	+ DMBA	OTD
10	-	DEHP (2,500 ppm)		OTD
11	-	DEHP (5,000 ppm)		OTD
12	-	DDT (50 ppm)		OTD
13	-	CBZ (500 ppm)		OTD
14	-	CBZ (1,000 ppm)		OTD
15	DMBA	DEHP (2,500 ppm)		
16	DMBA	DEHP (5,000 ppm)		
17	DMBA	PCB (100 ppm)		
18	DMBA	PCB (500 ppm)		
19	DMBA	DDT (25 ppm)		
20	DMBA	DDT (50 ppm)		
21	DMBA	CBZ (500 ppm)		
22	DMBA	CBZ (1,000 ppm)		
23	DMBA	OTD		
24	-	DEHP (2,500 ppm)		
25	-	DEHP (5,000 ppm)		
26	-	DDT (25 ppm)		
27	-	DDT (50 ppm)		
28	-	CBZ (500 ppm)		
29	-	CBZ (1,000 ppm)		
30	-	OTD		

[1]Embryo exposure groups will be started with 200 embryos, other groups with 100 healthy swim-up fry.
[2]Modulators were fed continuously from onset of feeding until termination.
[3]OTD = Oregon Test Diet.

significant binding of BP metabolites to hepatic DNA of trout occurred *in vivo*. The low proportion of apparent thioether conjugates present in bile indicates that detoxication of reactive BP metabolites may not be a major pathway in trout and that this inability to detoxify electrophilic metabolites may be related to the susceptibility of trout liver to BP-induced hepatocarcinogenesis.

Preliminary metabolism studies were conducted to determine the effect of CBZ, PCB, DEHP, and DDT on DMBA-DNA binding *in vivo*. Groups of 30 fry were fed control or test diets for 8 weeks. The pretreatment groups were: control, 1,000 ppm CBZ, 500 ppm PCB, 5,000 ppm DEHP, and 50 ppm DDT. DMBA in DMSO was administered by intraperitoneal injection; each fish received 2 mg DMBA/kg body weight (25 μCi [^{3}H]DMBA/fish). Liver DNA was isolated by phenolic extraction and specific activity determined. Values shown in Figure 1 are mean ± SE of three groups of 8 to 10 pooled livers.

These preliminary results show that PCBs increased DMBA-DNA binding while CBZ, DEHP, and DDT all decreased DMBA binding. It will be interesting to see if these results accurately predict the tumor response in the contaminant pretreated, DMBA fry-exposed experiment described above.

SPECIFIC AIM 3

Early efforts to develop a histochemical procedure to identify preneoplastic as well as neoplastic lesions in trout hepatocellular carcinogenesis have centered on the property of iron exclusion by carcinogen-altered cells (Williams and Yamamoto, 1972).

Twenty-one-day-old trout embryos were exposed to 100 ppm aqueous N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for 30 minutes. The fish were fed a semipurified diet and sampled monthly from the fourth to the ninth (end) month. Two days before sampling, the fish were iron-loaded with one intraperitoneal dose of 0.30 mg iron/100 g body weight as the dextran. Livers and kidneys were conventionally processed to paraffin sections for iron (Prussian blue), or hematoxylin and eosin (H&E) staining.

Normal hepatocytes accumulated iron in pericanalicular locations, but hepatocytes in carcinogen-altered foci and tumors exhibited a readily detectable resistance to iron accumulation. Normal renal tubule cells exhibited slight to moderate iron staining, while those from nephroblastoma were iron resistant. Renal hematopoietic tissue readily accumulates iron, so the deficiency of this tissue in nephroblastomas contributed greatly to the overall paucity of iron in the neoplasms.

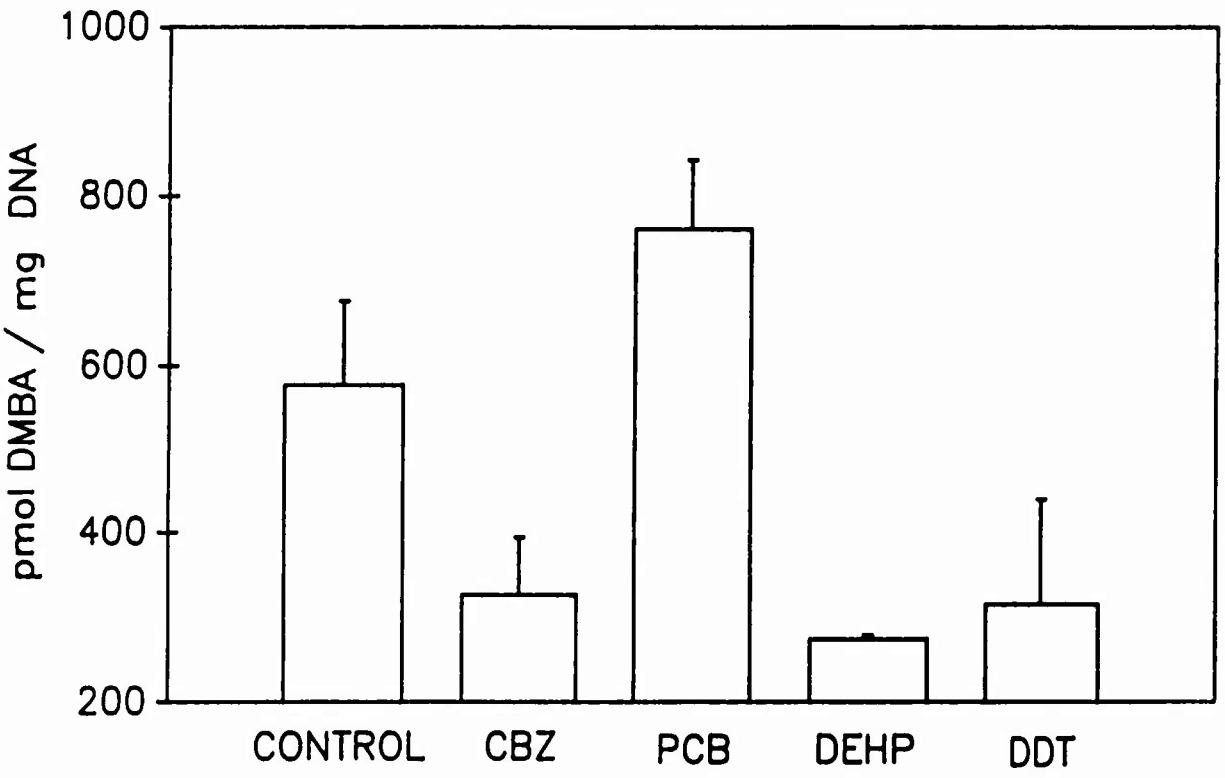


Figure 1. Effect of CBZ, PCB, DEHP, and DDT on DMBA-DNA binding in liver of trout.

Prior to these findings, the resistant property of carcinogen-altered hepatocytes to experimental iron loading has been used as a diagnostic tool in rodents only. The results with MNNG indicate that this property is present in carcinogen-altered hepatocytes of trout as well. Because early hepatocellular lesions in trout appear analogous to the preneoplastic foci found in rodent models, it is believed that iron resistance can become a diagnostic tool in the trout tumor model and perhaps in other fish species as well. Other carcinogens, i.e., aflatoxin B_1 (AFB1) and diethylnitrosamine, also produce iron-resistant hepatocellular lesions in trout (Nunez et al., 1989), so the method appears to be generally applicable to studies with chemical carcinogens.

The histological detail in Prussian blue sections was not sufficient to classify the iron-resistant lesions. Small, normally iron-resistant features, i.e., bile ductules and lymphocyte aggregations, were not always readily distinguishable from altered foci under Prussian blue staining. On the other hand, the histological alterations of preneoplastic lesions detected by iron resistance can be very subtle and undetectable under H&E alone, as Williams and Yamamoto (1972) noted in rats. Therefore, the combined use of Prussian blue for the detection of iron-resistant lesions followed by H&E semi-serial sections for classification offers a stronger diagnostic capability than either stain by itself.

The gross observation of lighter colored kidney tumors was histochemically confirmed as iron-resistant nephroblastoma. This characteristic has not previously been shown in neoplastic lesions of organs other than liver. Other kidney tumor types, e.g., cystadenoma, renal tubule cell carcinoma, or renal mesenchymal tumor, can be induced by chemical carcinogens. However, only nephroblastoma was present to examine for iron resistance in the trout. As greater numbers of renal tumors are examined, it may become possible to predict that all nephroblastomas will show iron resistance.

Current efforts on this specific aim involve the sequential sampling of fish following fry static water exposure to AFB1, methylazoxymethanol acetate (MAM-Ac), and diethylnitrosamine. H&E, iron staining, and several histochemical assays (glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, and Ca^{++} and Mg^{++}-dependent ATPase) are being used to characterize the progression of neoplasia caused by each of these carcinogens.

In addition, groups of inbred rainbow trout were exposed as fry to AFB1, MNNG, and MAM-Ac to initiate high incidences of liver, kidney, and swim-bladder neoplasms, respectively, for transplantation experiments into isologous inbred trout. Another group is being fed MNNG to initiate stomach tumors for transplantation.

SPECIFIC AIM 4

Initial attempts to obtain trout c-*onc* genes either by (1) probing Southern transfers of restriction enzyme digests of trout liver DNA or (2) probing two separate trout genomic libraries with a c-Ha-*ras* probe did not result in finding the genes. Therefore, the approach was changed by first amplifying a gene sequence *in vitro* by using polymerase chain reaction (PCR) (Saiki et al., 1988; McMahon et al., 1987). Two synthetic and degenerative oligonucleotide sequences (one corresponding to the first eight codons of a consensus mammal/goldfish *ras* sequence, and one derived from the complementary strand at codons 65 to 72 of the same consensus sequence) were synthesized for use as primers in the PCR procedure. Genomic DNAs from mouse NIH 3T3 cells, goldfish, and trout embryos or liver tissue were used. Due to a permissive annealing temperature (37°C) and varying elongation times (2 to 5 minutes), several bands were observed on gels of the amplified DNAs (Figure 2a). Probing a Southern transfer of the amplified DNAs with either a 20-base oligonucleotide synthetic c-Ha-*ras* probe or a short human c-Ha-*ras* sequence containing the first and second exons as well as the first intron, a band of approximately 800 base pairs in length believed to be a part of a trout *ras* gene was identified (Figure 2b). Isolation of this band is currently in progress.

At the same time, the primers were also used in the PCR procedure with the first strand of cDNA synthesized from total trout liver RNA. A single 216-base-pair band was observed on a gel. This band could be probed on a Southern transfer with a human c-Ki-*ras* cDNA probe but not with the human c-Ha-*ras* probes (Figures 2c and 2d). This fragment of DNA has been isolated and used to probe Southern transfers of restriction enzyme digests of trout liver DNA. Various bands were observed (Figures 2e and 2f). These fragments are currently being isolated and cloned to obtain the entire gene. The 216-base-pair piece has been sequenced, and the sequence is nearly identical to the human c-Ki-*ras* sequence.

DNA was prepared from 38 tumors collected from trout exposed to AFB1, MNNG, or DMBA. Fourteen of these DNAs were transfected into NIH 3T3 cells (Table 4). Colonies from several of the transfections were isolated and further grown, and cells were frozen for future assay.

Cells from four of the colonies were then grown for DNA isolation. The isolated DNA from colonies of samples 1, 2, 4, and 13 was digested with Hind III, run on an agarose gel, transferred to Zeta-probe membrane, and probed with a small trout DNA repetitive BamHI fragment for the presence of trout DNA in the transfected cells. The results were negative. Five colonies from tumor 28 transfection, which gave a high incidence of transformation, are

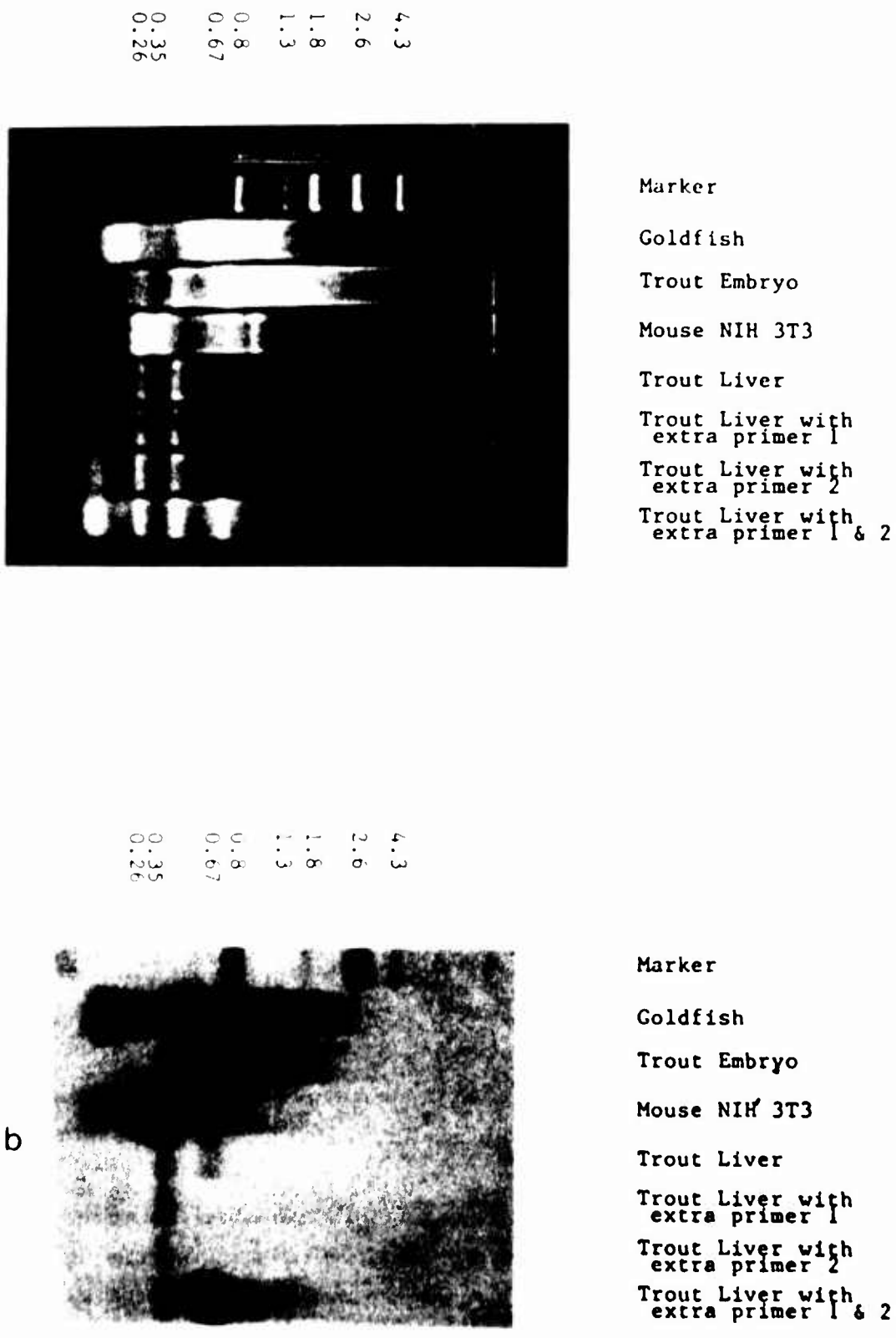


Figure 2. Identification of the trout *c-ras* gene by the PCR amplification method.

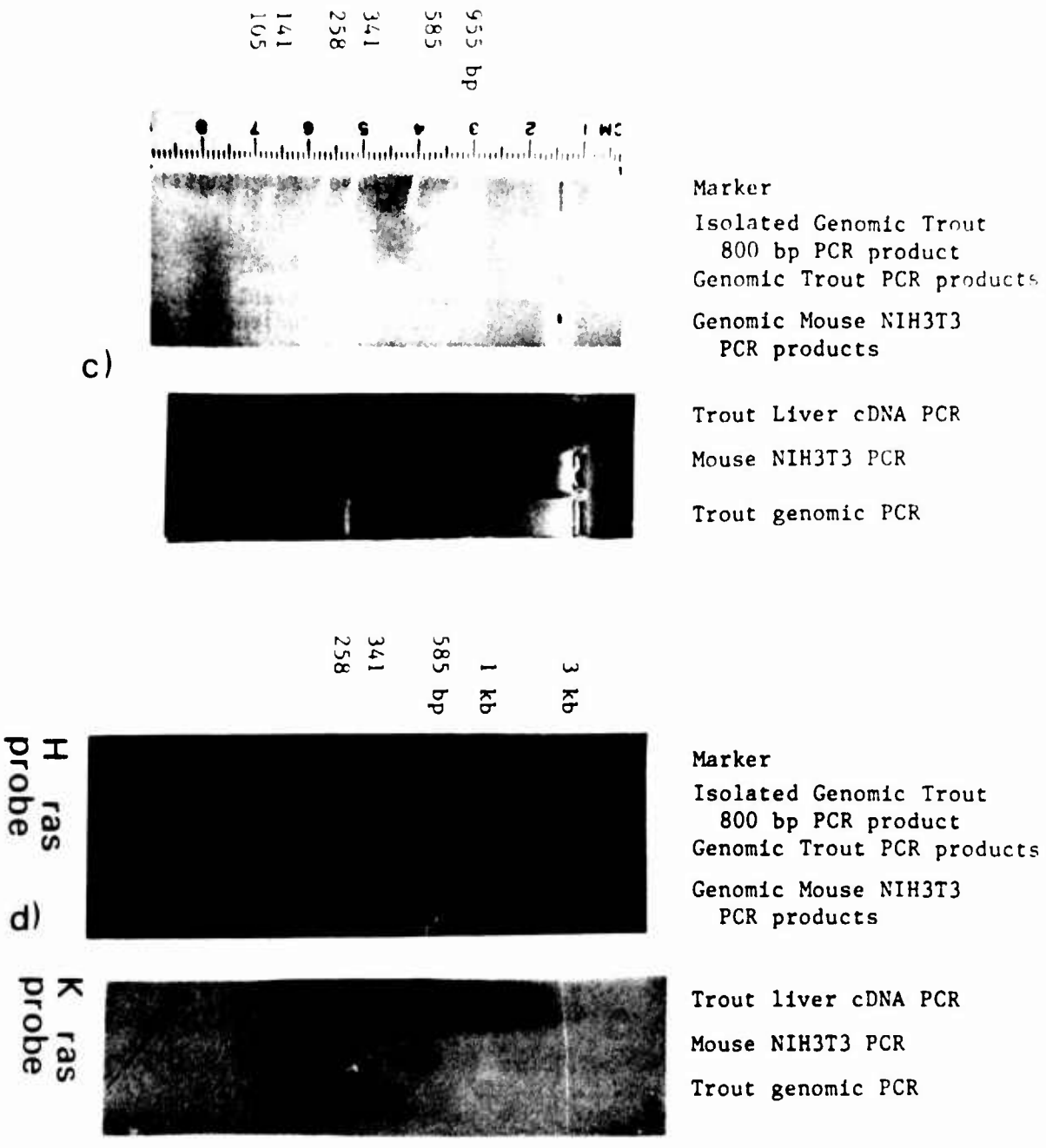
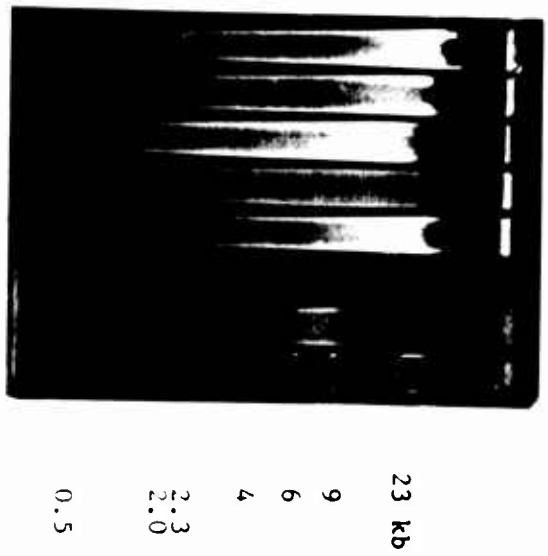


Figure 2. . Continued.

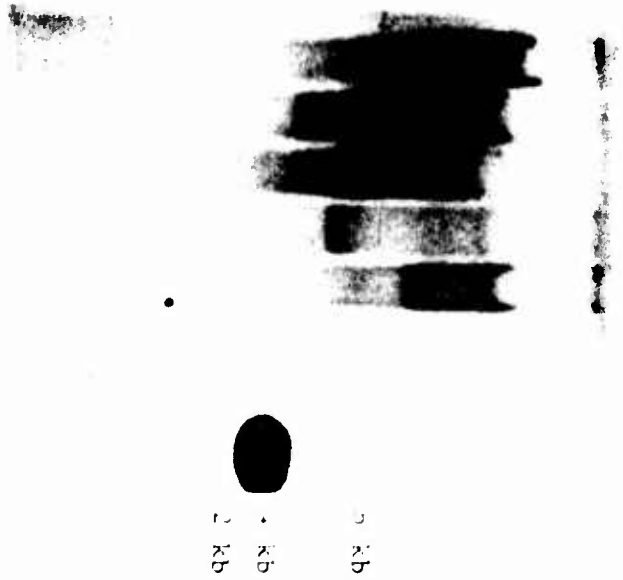
e



Trout Tumor #28 BamHI
Trout Tumor #28 EcoRI
Trout Tumor #28 HindIII
Trout Liver HindIII
Trout Liver BamHI
Trout Liver BamHI 6 kb sucrose gradient fraction
Trout Liver BamHI 9 kb sucrose gradient fraction
Marker (inc. 4kb K-ras and 3 & 1 kb H-ras markers)

23 kb
9
6
4
2.3
2.0
0.5

f



Trout Tumor #28 BamHI
Trout Tumor #28 EcoRI
Trout Tumor #28 HindIII
Trout Liver HindIII
Trout Liver BamHI
Trout Liver BamHI 6 kb sucrose gradient fraction
Trout Liver BamHI 9 kb sucrose gradient fraction
Marker

Figure 2. Continued.

Table 4. Summary of NIH 3T3 Transfections with Trout Tumor DNA

Sample #	Carcinogen*	Tissue	Foci/10 Plates	Foci/μg DNA
1st Transfection:				
1	AFB1	liver	6	0.12
2	AFB1	liver	4	0.08
3	AFB1	liver	0	--
4	AFB1	liver	4	0.08
	-control	A431 DNA	2	0.04
	+control	T24 DNA	16	0.32
2nd Transfection:				
8	MNNG	kidney	2	0.04
9	MNNG	kidney	0	--
10	MNNG	swim bladder	2	0.04
11	DMBA	liver	0	--
12	DMBA	liver	0	--
13	MNNG	liver	6	0.12
14	MNNG	liver	0	--
15	MNNG	liver	4	0.08
	-control	mouse liver	2	0.04
	+control	T24 DNA	8	0.16
3rd Transfection (repeat of #1, #2, #4, #13, #15):				
1	AFB1	liver	4	0.08
2	AFB1	liver	3	0.06
4	AFB1	liver	2	0.04
13	MNNG	liver	17	0.34
15	MNNG	liver	19	0.38
	-control	mouse liver	8	0.16
	+control	A427 DNA	18	0.36
4th Transfection:				
26	AFB1	liver	5	0.10
28	AFB1	liver	22	0.44
	-control	mouse liver	7	0.14
	+control	T24 DNA	13	0.26

*Abbreviations: AFB1--aflatoxin B_1 ; MNNG--N-methyl-N'-nitro-N-nitrosoguanidine; DMBA--7,12-dimethylbenz(a)anthracene

currently being tested. DNA from this same tumor (#8) was also transfected in a serum-free transfection system, and one colony was isolated. This colony was grown and DNA transfected a second time into the serum-free transfection systems, resulting in the appearance of several colonies.

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PATHOLOGY OF DIETHYLNITROSAMINE IN THE MEDAKA (*Oryzias latipes*)

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INTRODUCTION

The medaka (*Oryzias latipes*) is a small (3.0 to 3.5 cm long) aquarium fish native to Japan that has been considered attractive in the development of aquatic bioassays for carcinogenicity and toxicity testing because: (1) large numbers can be utilized in a small space, (2) it is a hardy fish and can be easily maintained and bred, (3) males and females are easily distinguished by external features, and (4) it is highly sensitive to known carcinogens (Klaunig et al., 1984). Diethylnitrosamine (DEN), a known carcinogen, has been shown to produce a variety of neoplastic and non-neoplastic lesions in the liver of the medaka (Hinton et al., 1988a,b). However, most studies using the medaka or other species have utilized adult animals exposed to relatively low levels of DEN for prolonged periods of time (Couch and Courtney, 1987; Ishikawa and Takayama, 1979; Ishikawa et al., 1975).

This report is part one of a two-part study, the second half of which was to sequentially study normal hepatic development in the medaka from prehatching stages to 60 days posthatch. The determination of normal developmental processes and cell types is important in order to assess pathologic processes that may occur secondary to carcinogenic exposure. The objectives of the study were to contribute data toward the establishment of the fish bioassay for carcinogenicity and toxicity testing by exposing the medaka to DEN, and to begin to determine the pathogenesis and progression of the hepatic lesions produced through interim sacrifices; to assess the use of a short-term exposure protocol (48 hours), which can be easier to perform than more prolonged assays and can reduce exposure risk to laboratory personnel; and to determine the effect of exposure level on lesion incidence and severity by using relatively low, medium, and high exposure levels.

MATERIALS AND METHODS

Medaka (*Oryzias latipes*) were housed and raised in 25°C tanks in accordance with established standards in the Health Effects Research Branch at the U.S. Army Biomedical Research Laboratory at Fort Detrick, Maryland. At 14 days of age, the fish were randomized

into four groups for DEN exposures of 100 mg/liter (group I), 200 mg/liter (group II), 400 mg/liter (group III), and 0 mg/liter (control). The total numbers of fish per group used for the study were n = 79 for group I, n = 140 for group II, n = 75 for group III, and n = 73 for the control group. Interim sacrifices were taken at 17, 31, 46, 59, 94, and 191 days postexposure (PE). Tissues were placed in Bouin's fixative for routine methacrylate embedment and staining, and in 4.0% cold glutaraldehyde in 0.1M cacodylate buffer for electron microscopy. Sections were taken at three to four levels through each liver and stained with a general stain of basic fuchsin/methylene blue. Special stains used included PAS and Gomori's methenamine silver. Tissues for electron microscopy were routinely processed for Araldite embedment and examined using a JEOL 100S electron microscope.

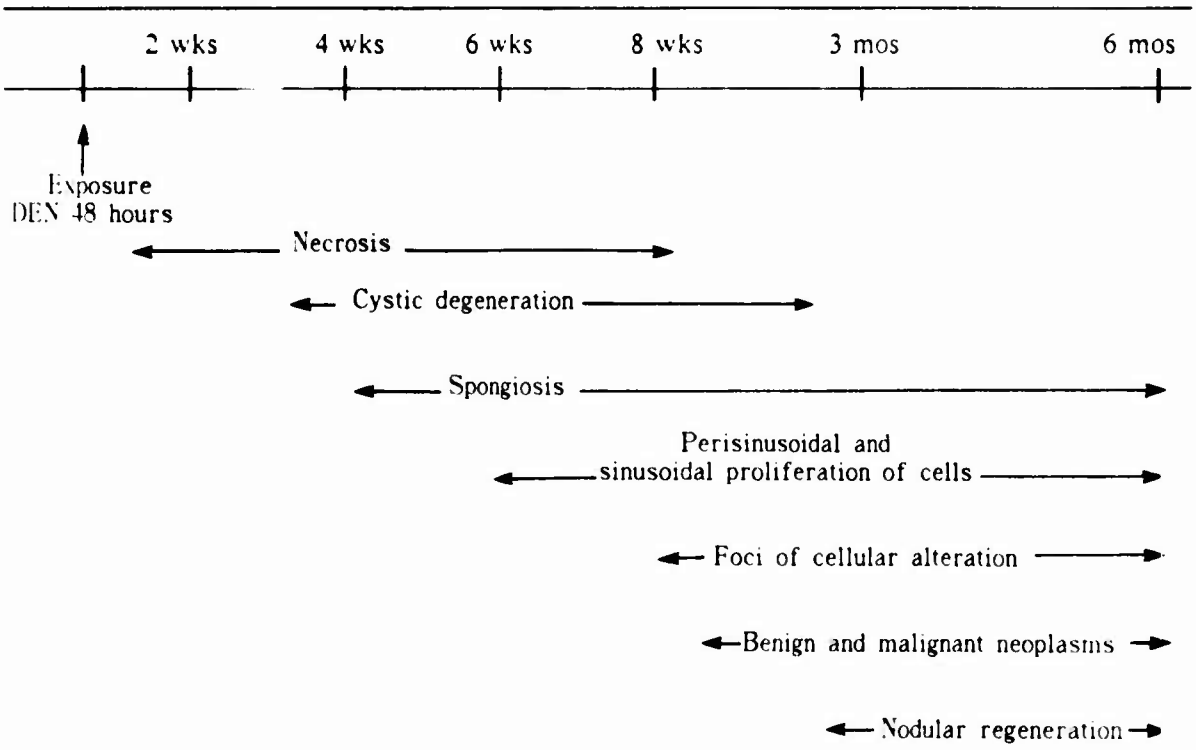
RESULTS

Details of this study are reported in depth elsewhere (Bunton, 1989b) but are summarized here. Table 1 shows the progression of changes seen: an early phase of cellular necrosis and cystic degeneration of the hepatic parenchyma followed by various proliferative and neoplastic changes. The relationship between the exposure level of DEN and the incidence and severity of many of the lesions was direct. This was particularly true in comparisons between the low-level exposure group and the two higher exposure levels as the latter two groups exhibited many similarities. However, the highest exposure level produced the most profoundly affected livers. In group I (100 mg/liter DEN), lesions often involved a minimal portion of the liver and more fish throughout the study had histologically normal livers. One hepatic malignancy was seen in this group. By contrast, lesions in group III (400 mg/liter) were often complex and extensive and only a few fish at any time during the study had histologically normal livers. Several malignant hepatic tumors were seen in this group. Changes in group II (200 mg/liter DEN) fell somewhere between groups I and III.

Non-neoplastic lesions seen throughout the study were eosinophilic cytoplasmic inclusions (membrane bound, nonfibrillary cytoplasmic bodies compatible with lysosomes); spongiosis hepatis, which was similar histologically to what has previously been reported in rats (Bannasch et al., 1981) and fish (Hinton et al., 1988a; Couch and Courtney, 1987); and cellular vacuolation, which seemed most commonly to represent fatty change.

Foci of cellular alteration were seen 45 days PE, were primarily basophilic, and were enlarged in some fish in groups II and III to be more appropriately called areas of cellular alteration. Cholangiofibrosis and nodular regeneration were also seen as a later change in a few fish.

Table 1 Progression of Hepatic Lesions Seen After 48-Hour Exposure to DEN



From Bunton (1989a).

A list of benign and malignant neoplasms is shown in Table 2. There was only one neoplasm in group I: a well-differentiated hepatocellular carcinoma. The other groups showed a mixture of tumors with a predominance of biliary tumors and sarcomas. Although sample size per sacrifice was somewhat small, the total number of malignant neoplasms appeared to be directly related to exposure (group I, 1.7%; group II, 5.8%; group III, 10.9%; and 0% in controls).

Several cellular proliferative lesions were also seen. These were variable in appearance and were present from 46 days PE. They were present in perisinusoidal, sinusoidal, and peribiliary regions and consisted of spindle-shaped dendritic cells, angular cells with oval nuclei, cells that formed occasional acini, or rounded, individualized cells similar to macrophages. It is not known at this time if these areas represent precursors to some of the neoplasms seen, but they appeared to represent more than one cell type.

Table 2. Benign and Malignant Neoplasms in the Medaka

Type	Exposure Group and Number of Tumor Type
Adenoma	II (1), III (1)
Cholangioma	II (1)
Hepatocellular carcinoma	I (1), II (2)
Cystadenocarcinoma	II (1)
Cholangiocarcinoma	II (1), III (1)
Sarcoma with vascular orientation	II (1), III (2)
Histiocytic sarcoma*	II (1), III (1)
Carcinosarcoma	III (1)
Malignant - undetermined type	II (1), III (1)

*Tentative based on histology and ultrastructure.

CONCLUSIONS

Many of the lesions seen were similar to what is seen with adult, long-term DEN exposures. However, a difference may be the preponderance of biliary tumors and sarcomas. It will be necessary in the future to develop additional techniques for the identification of cell types in some of the proliferative and neoplastic changes seen, such as immunocytochemistry or enzyme histochemistry. The definitive identification of cell types will make it possible to begin to determine why certain protocols result in the development of certain types of neoplasms.

ACKNOWLEDGMENT

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BIOLOGICAL (MOLECULAR AND CELLULAR) MARKERS OF TOXICITY IN THE JAPANESE MEDAKA

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INTRODUCTION

The objective of this research is to evaluate the Japanese medaka as a model for prediction of potential health effects from environmental contaminants. Progress to date has concentrated on efforts to describe the molecular and biochemical events resulting from exposure to a well-characterized model carcinogen, diethylnitrosamine (DEN). A fundamental understanding of the molecular and biochemical events underlying the carcinogenesis process in medaka may make it possible to employ these responses as biomarkers to estimate exposure and predict cellular effects of genotoxic chemicals. Thus, it may be possible to determine if an individual chemical, or waste effluent from Department of Defense facilities, poses a health threat based on exposure of medaka to the contaminant for the short time required for the biomarker to respond, rather than waiting for the 6 months required to evaluate a histopathological effect. More fundamentally, critical evaluation of the molecular carcinogenesis process in medaka provides a foundation for using this convenient and cost-effective animal model for more directly extrapolating to human health effects, based on pharmacodynamic models. Before this approach can be accepted, it is necessary to demonstrate that the molecular, biochemical, and cellular events in carcinogenesis are similar in fish and humans. If the results of this research support such a conclusion, a later task of this project will involve development of a pharmacodynamic model for extrapolating human health effects from bioassays using the Japanese medaka.

This research has focused on two key biochemical processes: (1) DNA alterations, and (2) activity of enzymes that metabolize the chemicals to highly toxic intermediates and also to readily excreted polar compounds. Two phases of metabolism are recognized, and enzymes representative of both phases have been examined in this research. In Phase I metabolism, catalyzed by the mixed function oxidase (MFO) enzyme system, a reactive functional group (such as -OH) is inserted in the parent compound. This enhances the water solubility and, hence, excretability of the substrate; moreover, these activities provide suitable substrates for

Phase II conjugating enzymes described below. MFO-mediated reactions are considered important detoxification pathways. However, in a number of instances including key environmental contaminants, this pathway serves to activate substrates to more reactive, toxic products. For example, procarcinogens are metabolized to DNA-reactive carcinogens via MFO activities. Phase II enzymes catalyze the conjugation of highly polar endogenous compounds (generally based upon glucose, amino acids, or sulfate) onto suitable substrates, such as Phase I metabolites of lipophilic contaminants. The reactions comprising Phase II metabolism serve to detoxify carcinogens via greatly enhanced excretability of the products. The enzyme receiving attention in this study is the glutathione S-transferase (GST), which catalyzes conjugations with reduced glutathione (GSH).

Because alteration of the DNA is understood to be the initiating event in carcinogenesis, the effect of DEN on several types of DNA damage are being evaluated in the medaka, including early events such as adduct formation and secondary modifications of DNA integrity, as well as later, generally irreversible effects such as cytogenetic aberrations.

METHODS AND MATERIALS

Exposure Protocol

Approximately 70 adult medaka (both sexes) were exposed by the U.S. Army Biomedical Research and Development Laboratory (USABRDL) to a solution of 200 mg/liter of DEN for 24 hours, followed by transfer to clean water for 6 days. This exposure protocol was repeated three additional times for a total of four incremental exposures to DEN. A control population of 70 fish was similarly treated, but without exposure to DEN. At the end of the exposure, 10 fish from each group were retained by USABRDL for histopathological evaluation. The remaining animals were sent to Oak Ridge National Laboratory for analysis. Fish in the exposed and control groups were pooled in groups of 10 animals (approximately equal numbers of each sex in each replicate). Livers were removed and pooled for evaluation of detoxication enzymes and measurement of DNA alterations (strand breaks and abnormal DNA distribution). The remainder of the animals (termed "carcass") in each replicate was pooled for additional measures of DNA alteration (strand breaks, adducts, and minor nucleoside content).

DNA Alterations

Several measures of DNA alteration were evaluated.

DNA Strand Breaks

DNA isolation was accomplished by homogenizing the intact medaka in 1 *N* NH_4OH/0.2% Triton X-100. The DNA was further purified by differential extraction with chloroform/isoamyl alcohol/phenol (24/1/25-v/v) and passage through a molecular sieve column (Sephadex G50).

DNA strand breaks were measured in the isolated DNA by an alkaline unwinding assay as modified by Shugart (1989). The technique is based on the time-dependent partial alkaline unwinding of DNA followed by determination of the duplex:total DNA ratio (*F* value). This procedure has been further modified to accommodate the isolation and detection of strand breaks in the DNA from a single medaka liver.

The relative number of strand breaks (*N* value) in DNA of medaka from an exposed population can be compared to those from a control population as follows:

$$N = (\ln F_e / \ln F_r) - 1$$

where F_e and F_r are the mean *F* values of DNA from the exposed and control populations, respectively. *N* values greater than zero indicate that DNA from the exposed population has more strand breaks than DNA from the control population; an *N* value of 5, for example, indicates five times more strand breakage.

Minor Nucleoside Content (5-Methyl Deoxycytidine)

Deoxyribonucleoside analysis was performed by a modification of the procedure of Uziel et al. (1965) on DNA isolated from one intact medaka. To approximately 25 μg of DNA in buffer, 10 μg each of pancreatic DNase, snake venom phosphodiesterase, and bacterial alkaline phosphatase was added. The contents were mixed and incubated at 37°C for 1 hour. The mixture was injected onto a 0.6- × 45-cm glass column packed with the cation exchanger Aminex A-6 (Bio-Rad Labs, Richmond, CA) equilibrated with 0.45M ammonium formate, pH 4.5. The column was maintained at a constant temperature of 50°C, and the sample was eluted isocratically in the same buffer at a flow rate of 1.0 ml/minute. The column eluent was monitored by an absorbence detector at 260 nm and the chromatographic data recorded.

DNA Adducts (Monoclonal Antibodies)

This work was performed in collaboration with Dr. James M. Parry, University College of Swansea, Wales, United Kingdom. O^6-Ethyl guanine adducts in DNA were detected by a

noncompetitive solid-phase immunoassay (Adamkiewics et al., 1985). Approximately 3 μg of single-stranded DNA, produced by heat denaturation, were immobilized on nitrocellulose filters and any O^6-ethyl guanine residues were detected with monoclonal antibodies.

Abnormal DNA Distribution of Hepatocytes (Flow Cytometry)

This work was conducted in collaboration with Dr. Joseph E. Fuhr, Memorial Research Hospital, University of Tennessee, Knoxville, Tennessee. Liver tissue was prepared for flow cytometric analysis according to the procedure of McBee and Bickham (1987). DNA histograms indicating the frequency of DNA distribution within the hepatocytes were recorded for each preparation.

Detoxication Enzymes

Phase I Activity (Ethoxyresorufin-O-deethylase)

Livers of fish were pooled, weighed, and homogenized in sucrose 0.25 M, 0.1 M Tris buffer at pH 7.4. Homogenates were centrifuged twice and microsomes obtained by differential centrifugation at 106,000 x g for 2 hours. Microsomes and cytosolic supernatants were stored at -120°C until used for the enzyme activity assays.

The activity of 7-ethoxyresorufin-O-deethylase (EROD) from the low-speed supernatant of the liver homogenate and from the purified hepatic microsomes was measured fluorometrically at 30°C (Burke and Mayer, 1974) and expressed as pmoles of resorufin min^{-1} mg^{-1} of microsomal and homogenate protein, respectively.

Concentrations of cytochrome P450 in microsomes were determined spectrophotometrically by a modification of the methods of Omura and Sato (1964). Cytochrome P450 was oxidized with carbon monoxide and reduced with sodium dithionite. The concentration of cytochrome P450 is expressed as nanomoles of cytochrome P450 per mg of microsomal protein.

Phase II Activity (Glutathione-S-transferase)

Glutathione-S-transferase (GST) activity was determined on cytosol fractions according to the methods of Habig (1974). GST activity was estimated with glutathione reduced form (GSH, 1 mM) and 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate in a spectrophotometer at 340 nm. The results were expressed in units of enzyme activity per mg of protein. Microsomal and liver homogenate proteins were determined using a centrifugal fast analyzer (Cobas Fara) with the Bio-Rad protein assay reagent. Bovine serum albumin was used as the standard. The protein concentration was expressed as mg/ml.

RESULTS AND DISCUSSION

Evaluation of Detoxication Enzymes

Assay conditions for the enzyme assays were optimized with respect to substrate concentration, pH, and temperature using adult fish that had not been exposed to any chemicals. Optimal conditions are listed in Table 1. The Km for the EROD activity was determined and was in the same range as has been reported for the rat and for a number of other fish species (Table 2).

EROD purification and recovery were evaluated by comparing activity assayed in the low-speed supernatant (referred to as the "homogenate" fraction) and the activity recovered in the purified high-speed supernatant (the microsome fraction). As indicated in Table 3a, although the differential centrifugation purified the protein threefold, EROD activity in the microsomes was only sightly greater than that observed in the low-speed supernatant. The budget of activity and amount of protein showed that over half of the total EROD activity was lost during the purification. This could be due to several reasons, including enhanced denaturation of the protein because of the small volumes and dilute protein concentrations dictated by the size of medaka livers. Cytochrome P450 spectra indicated a large peak at 420 nm, relative to the peak at 450 nm, suggesting degradation of the cytochrome P450 during purification. The possibility that the cytosol contains an endogenous inhibitor of EROD activity was also tested. Microsomes were isolated and diluted with an equal volume of either the high-speed supernatant (the cytosol) or with an equal volume of assay buffer. Results of three EROD assays are shown in Table 3b. EROD activity was 18 to 38% lower in the presence of the cytosol, suggesting the possibility of a cytosolic inhibitor. These results suggest that for this study measurements of EROD activity be taken in the low-speed supernatant until problems of microsomal purification can be resolved.

EROD and GST activity were measured in livers of the medaka exposed to the DEN and in the control group. EROD activity decreased significantly in the DEN-exposed group (Table 4); the magnitude and direction of the decrease were similar regardless of whether the enzyme activity was measured in the low-speed supernatant or in the purified microsomes. In contrast, GST activity increased over 40%, although the statistical significance of that increase was marginal (Table 4). The general pattern of depressed Phase I activity and enhanced Phase II activity following exposure to genotoxic agents such as DEN has also been observed in rodents.

Table 1. Optimum Conditions for Various Enzyme Assays

		EROD FOR LIVER HOMOGENATES		
Buffer	pH	[7-ethoxyresorufin]	Assay temp.	[NADPH]
Tris 0.1 M	8.0	1.5 μM	30°C	0.3 mM
		EROD FOR LIVER MICROSOMES		
Buffer	pH	[7-ethoxyresorufin]	Assay temp.	[NADPH]
Tris 0.1 M	8.0	1.5 μM	30°C	0.3 mM
		GSH-T FOR CYTOSOLIC FRACTIONS		
Buffer	pH	[GSH]	[CDNB]	Assay temp.
Phosphate 0.1 M	7.4	1 mM	1.5 mM	30°C

Table 2. EROD Activity in Livers of Rat and Fish Species

Species	Km (μM)
Medaka	0.11
Croaker	0.36
Hogchoker	0.19
Toadfish	0.16
Scup	0.24
Rainbow Trout	0.14
Rat	0.13-0.16

Table 3a. EROD Purification and Recovery

Step	EROD		Protein
	(Activity)	(Units)	(Recovery)	(Recovery)
Low Speed	8.65	32.48	100%	100%
Microsomes	11.47	13.73	42%	33%

Table 3b. Test for EROD Inhibitor in Cytosol

Sample	EROD		Inhibition
	(Microsomes)	(+Cytosol)	(%)
A	12.67	10.41	17.8
B	34.52	23.33	32.4
C	19.91	12.34	38.0

Table 4. Summary of Results

Detoxication Capacity

Group	EROD[a]	GSH-t[b]
Control	8.22±3.76 (5)	534±88 (5)
DEN-Exposed	2.70±1.16 (5)	724±213 (6)

DNA Alterations

Group	Strand Breaks[c]	5m-dCyd	DNA Distribution
Control	0.40±0.04 (10)	4.8	normal
DEN-Exposed	0.21±0.12 (30)	5.5	tetraploidy

t-test:

[a] alpha > 0.05 < 0.001

[b] alpha > 0.01 < 0.05

[c] alpha > 0.001

DNA Alterations

DNA Strand Breaks

Because of the limited amount of liver tissue, an attempt was made to isolate DNA from the low-speed pellet in the same samples being used to measure EROD and GST activity. Unfortunately, the conditions of the homogenization were sufficiently severe to shear the DNA polymer and make it impossible to quantify the number of strand breaks caused by the exposure to DEN.

Strand breaks were evaluated in DNA isolated from the medaka carcasses and are reported as the *F* value in Table 4. There is a slight, but statistically significant, decrease in the duplex:total DNA ratio of the DEN-exposed group, indicating an increased number of strand breaks. Based on these *F* values, the relative number of strand breaks (*N*) in the DEN-exposed fish is 0.7, that is, the DNA of the DEN-exposed animals has 70% more strand breaks than the DNA from the control animals. This is a relatively small difference in DNA integrity, compared to fish collected from contaminated streams or exposed to low (1 μg/liter) concentrations of benzo(a)pyrene (BaP) (Shugart 1990), in which *N* values can reach levels of 6 to 10 (i.e., 6- to 10-fold increases in number of strand breaks).

Minor Nucleoside Content

The only methylated deoxynucleoside in eukaryotic DNA is 5-methyl deoxycytidine (5m-dCyd). It has been demonstrated in cell culture that chemical carcinogens and mutagens alter the normal patterns of DNA methylation by interfering with the fidelity of the normal postreplicative modification of the DNA. The hypomethylation of the DNA has been shown to lead to inheritable abnormalities in gene expression. The effect of carcinogens on DNA methylation patterns has been demonstrated *in vivo* in fish. Bluegill sunfish exposed to BaP (1 μg/liter) for 40 days experienced a 50% decline in 5m-dCyd content in their DNA.

Analyses of 5m-dCyd content of DNA from medaka carcasses are in progress. The results of the single pair of samples that has been analyzed does not reveal any substantial difference in exposed versus control fish (Table 4).

DEN-DNA Adducts

A DNA adduct is a chemical or its metabolite that is covalently bound to DNA. Modification of the DNA by adducts is understood to be a critical event in carcinogenesis and mutagenesis. A wide range of specific ethylated adducts form on DNA following exposure to DEN, and protocols for isolating and quantifying these adducts using HPLC have been well

described. Because these procedures are time consuming and labor intensive, an alternate method for detection of DEN adducts of DNA--the use of monoclonal antibodies to the adducts--has been explored in this research. The advantages of the antibody approach include: (1) adequate sensitivity (adduct levels of 1 adduct per 10^6 to 10^8 can be readily detected); (2) cost effectiveness (large numbers of samples can be analyzed simultaneously); and (3) selectivity (levels of a specific adduct can be quantified in the presence of other adducts). Potential problems include concerns that antibody specificity may be compromised because the DNA structure may sometimes be recognized by the antibody, but this can be addressed by enzymatic degradation of the DNA prior to introduction of the antibody.

Samples of purified DNA were sent from Oak Ridge National Laboratory to Dr. Parry for analysis of O^6-ethyl guanine adducts, using the monoclonal antibodies he has developed. Of the array of ethylated DNA adducts formed by exposure to DEN, the O^6-ethyl guanine adduct was focused on because its concentration and persistence have been correlated with tumor formation in several species. Three replicate samples of DNA from the DEN-exposed animals and four samples from the control animals were assayed, and no O^6-ethyl guanine adducts were detected in any of the samples (detection limits are estimated to be approximately 1 adduct per 10^6 to 10^7 nucleotides).

Given these results, it is perhaps significant to note that this adduct can be enzymatically cleaved by the activity of the enzyme, O^6-alkylguanine-DNA alkylase, and that malignant transformation may be associated with activation of this enzyme. For many, if not most, adducts, including well-characterized carcinogens such as BaP, adducts are removed by excision repair; a long segment of DNA is removed along with the chemically modified nucleotide, and the excised region is then repaired by the activity of DNA polymerase and ligase. In this excision repair process, then, removal of adducts generates strand breaks in the DNA that will be detected by the alkaline unwinding procedure. In the case of the O^6-ethyl guanine adducts, however, adducts can be dealkylated enzymatically without causing strand breaks. Thus, it is possible that exposure of the medaka to DEN activated the alkyltransferase, which removed the adducts but did not result in increased levels of DNA strand breaks (Table 4).

Abnormal DNA Distribution in Hepatocytes

The distribution of DNA content in hepatocytes of exposed and control medaka was analyzed using a flow cytometer. The DNA content of cells is known to be altered by mutagens, carcinogens, and ionizing radiation. The use of a flow cytometer to measure the

DNA distribution of cells is recommended because of the rapid sampling, low cost of the analyses, and the large number of cells that can be analyzed with this instrument.

Results of the flow cytometer analyses can be interpreted within the context of the cell cycle. The majority of the cells are in the G1, or "resting," stage of the cell cycle. The DNA content of the cells represents the normal diploid complement of DNA in each cell. Cells in G2, the stage of cell cycle in which the DNA has replicated, but before the cell divides, are tetraploid and have twice the DNA content per cell as a G1 cell.

On a quantitative basis (Figure 1), the average number (percentage) of cells with DNA content beyond diploid G0/G1 was higher in the exposed medaka livers (18.9%) than was found in the medaka livers (12.8%).

Beyond quantitation, however, there were qualitative differences in the histograms in the two groups of fish (Figure 1). The descending side of the diploid G0/G1 population in the exposed fish was more diffuse and did not descend to baseline as clearly as in the control samples. This pattern change could be attributable to more cells moving into the early S phase of the cell cycle, or more likely represents the presence of reactive or inflammatory cells in the samples. Because the stain utilized in the analyses is an intercalating dye, it has been proposed that as cells become more metabolically active, their DNA unwinds, exposing more sites for dye to bind. This gives the appearance of increased DNA within the nucleus. For this reason, it is believed that the most significant difference, based on very few numbers, is the apparent increase in inflammatory cells in the exposed fish specimens.

The numbers of cells in the diploid G2 region also appear to be increased. This difference, however, will require additional studies for confirmation. Some species normally accumulate cells in G2, and the loss of cells in that region represents abnormalcy. More studies would be necessary to evaluate this observation in fish.

Histopathology of DEN-Exposed Medaka

The exposed and control medaka preserved for histopathological evaluation have not been analyzed at this time. These data will be useful in interpreting the possible causes of the abnormal DNA distribution and other data. It is unclear, for example, if the effects described are related to a carcinogenic effect, as evidenced by formation of preneoplastic foci or lesions, or whether the effects reflect a noncancerous cellular toxicity resulting from exposure to the very high levels of DEN. Cytotoxic effects have been shown to alter some of the molecular and biochemical responses measured in this study; for example, treatment of sunfish with hepatotoxic agents reduces contaminant-associated induction of EROD activity and results in increased levels of DNA strand breaks (McCarthy et al., 1989).

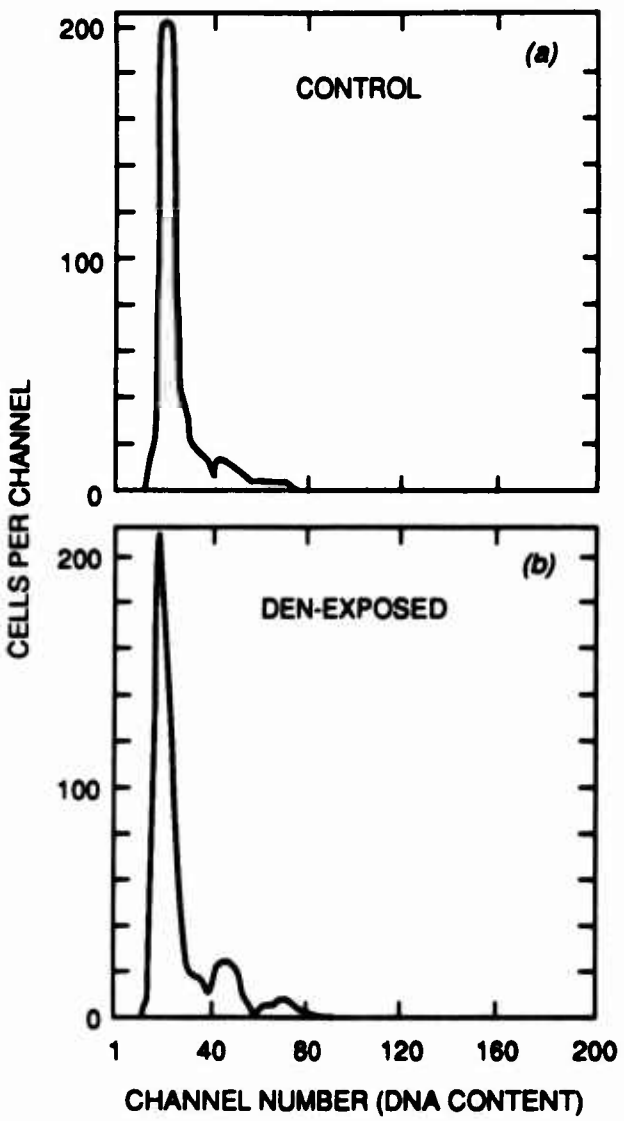

Figure 1. Frequency histograms of DNA content in hepatocytes. The results of analyses of medaka hepatocytes from (a) control animals and (b) DEN-exposed animals are shown. The major peak represents cells in the G1 (resting) stage of the cell cycle.

SUMMARY AND CONCLUSIONS

Several molecular and biochemical markers of toxicity have been adapted for measurement in medaka and have been applied to describe the effects of treatment of adult medaka with a high dose of DEN. As summarized in Table 4, DEN treatment inhibited Phase I enzyme activity and increased the activity of a Phase II enzyme; this pattern of response has been described in preneoplastic rodents. No O^6-ethyl guanine adducts were detected, and only a slight (but statistically significant) increase in DNA strand breaks was observed. Limited numbers of samples have not yet revealed any effects of DEN exposure on 5m-dCyd content of the DNA. These results are consistent with a hypothesis that the prolonged exposure and high levels of DEN induced alkyltransferase activity, which enzymatically removed any adducts that formed but did not result in strand breaks or hypomethylation of the DNA, such as might be expected from excision repair of chemically modified DNA. The DEN-exposed animals had a significantly greater fraction of hepatocytes in the G2 phase of the cell cycle, suggesting increased cellular replication. Histological evaluation of the DEN-exposed fish should help determine whether these effects can be attributed either to carcinogenic transformations or, alternately, to direct cytotoxic effects of the high dose of DEN.

ACKNOWLEDGMENTS

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ASSESSMENT OF DNA MODIFICATIONS IN MEDAKA

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INTRODUCTION

An urgent need exists for methods that provide definitive information on the effects of groundwater contaminants on animal systems. In particular, a requirement exists for methods that elucidate changes in DNA resulting from exposure to a broad range of environmental chemicals.

Accordingly, a study was initiated to establish protocols for identifying DNA damage in medaka (*Oryzias latipes*) exposed to free radical-forming chemicals. Gas chromatography, coupled with mass spectrometry and single-ion monitoring (GC/MS-SIM) of derivatized nucleotide bases, was the analytical approach of choice. The work of Dr. Miral Disdaroglu (1985) formed the basis of the experimental protocols. He is with the National Institute of Standards and Technology, Gaithersburg, Maryland.

RESEARCH OBJECTIVES AND METHODS

Basically, the task was to excise the livers of medaka, isolate the DNA in pure form, liberate the nucleotide bases by hydrolysis, and form trimethylsilyl derivatives. These derivatives would then be analyzed by GC/MS-SIM for evidence of base oxidations, such as 5-hydroxythiamine and 8-hydroxyadenine, both of which are products of free radical-induced interactions with the respective nucleotide bases. The data would also serve to delineate the site(s) where the free radical modifications had occurred in the nucleotide bases.

The first phase of the research required the isolation of sufficient medaka liver to make it possible to obtain relatively pure DNA in sufficient quantity to proceed with the preparation of derivatives for GC/MS-SIM. After several trials, it was determined that about 200 μg of DNA was ideal for the research. Initial attempts to identify the desired nucleotide derivatives by GC/MS alone were not successful, due primarily to interference from a wide spectrum of apparent breakdown products. However, application of the SIM mode provided evidence for small concentrations of a number of hydroxylated bases from DNA isolated from normal medaka. It was clear that these derivatives could be quantified if appropriate

standards were employed. Accordingly, reference compounds were synthesized, and some were obtained from commercial sources.

In the course of this work, it became apparent that an appreciable advantage would be gained if it were known which nucleotide base derivatives were most reflective of free radical-induced modifications. In fact, the greater the magnitude of the increase, the greater the chance of establishing differences between control and test samples. In this regard, it was fortunate to learn of Dr. Disdaroglu's recent findings with iron ion-dependent modifications of DNA bases, using the superoxide radical-generating system hypoxanthine/xanthine oxidase. In fact, his very recent *in vitro* studies demonstrated that certain nucleotide base derivatives, such as 2,6-diamino-4-hydroxy-5-formamidopyrimidine, which were present in normal DNA in almost imperceptible concentrations, were increased dramatically in the presence of the hypoxanthine/oxidase system (Aruoma, 1989).

Disdaroglu's findings were obviously important and timely for this research. Thus, an immediate modification was made in the analytical procedure to include a search for 2,6-diamino-4-hydroxy-5-formamidopyrimidine and other "sensitive" derivatives. At present, several of these derivatives are being synthesized for use in quantitation, while at the same time preliminary attempts are being made to establish their presence on the basis of reported spectral properties.

FUTURE EFFORTS

For the immediate future, the research will concentrate on methods for the quantitative analysis of a number of the modified nucleotide bases identified by GC/MS-SIM. Efforts will also continue to increase the supply of pure derivatives for this purpose, which may require synthesis in Pacific Northwest Research Foundation's laboratories. In addition, medaka livers will continue to be used for the isolation of DNA and the preparation of trimethylsilyl derivatives.

One of the immediate objectives is to positively identify, and ultimately quantify, modified base structures present in the DNA of normal medaka. These structures are expected to occur as a result of normal biochemical events, such as those associated with the repair process.

Also part of the schedule will be a request to the U.S. Army Biomedical Research and Development Laboratory for medaka exposed to single xenobiotics and mixtures present in contaminated groundwater. It is expected that analyses on these samples will be attempted in the near future.

Clearly, the success of future work will be dependent, to a large measure, on whether the compounds recently elucidated by Disdaroglu will increase in exposed medaka as substantially as they do in the *in vitro* system. Hence, a major effort will be oriented toward investigating this possibility in the months ahead.

Overall, the approximately 10 months of effort so far have been most productive, and the Foundation feels very positive about its ability to succeed with its objectives, including the ability to quantify differences between control and test samples by using several nucleotide derivatives reflecting free radical-induced damage to medaka DNA. Presently, over a dozen such nucleotide derivatives are being analyzed.

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ROLE OF ONCOGENES IN CHEMICAL CARCINOGENESIS

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ABSTRACT

In previous studies (Van Beneden et al., 1988), transfection studies were described whereby DNA isolated from chemically induced liver tumors in the Japanese medaka (*Oryzias latipes*) was introduced by calcium phosphate precipitation into mouse NIH 3T3 cells together with the plasmid pSV_2neo. DNA isolated from a diethylnitrosamine (DEN)-induced cholangiocarcinoma was highly transforming in primary, secondary, and tertiary transfection assays. Southern blot analysis of DNA isolated from transfected cells revealed fish-specific bands only in DNA digests from tumor-induced transfectants. There appeared to be no homology to known oncogene sequences. Parallel secondary and tertiary transfection experiments were performed without additional pSV_2neo sequences to determine the efficiency of the transformations. Southern blots of DNA digests from these foci cells, when hybridized to pSV_2neo under high stringency conditions, revealed homologous sequences present in transformed cells that were not present in control cells. These results suggest that pSV_2neo, or some portion of it, has integrated into the host cell genome at a site near that of the fish transforming gene. A library was prepared using *Sau* 3A I partial digests of DNA from these tertiary transfectants ligated into a λ DASH vector. This library is currently being screened for pSV_2neo sequences.

INTRODUCTION

While the detailed mechanisms of tumorigenesis are unknown, increasing evidence suggests that genetic alterations of cellular oncogenes are in part responsible for the neoplastic transformation of cells. Investigations using rodent models have implicated the direct activation of cellular oncogenes by carcinogen treatments (Barbacid, 1987). One of the best defined systems is the activation of H-*ras*-1 in nitrosomethylurea (NMU)-induced mammary carcinoma in Buf/N rats (Sukumar et al., 1983). The mutation involves a specific G→A transition of the second base of the 12th codon. NMU is known to induce such mutations by

methylation of deoxyguanosine at the O^6 position. The reproducible detection of specific transforming genes in animal model systems strongly suggests that these oncogenes have a significant role in the development of certain tumors. That the same type of activating mutation is observed in chemically induced tumors in animals as is seen in human tumors further validates the use of animal systems as models for human carcinogenesis.

While most of these studies have been done in rodent systems, nonmammalian models, especially teleost fishes, have recently become important in carcinogenicity studies. Fish tumor investigation is now seen as an integral part of the basic, biological approach to elucidating common mechanisms of carcinogenesis at different phylogenetic levels. A recent study by McMahon et al. (1988) reported a point mutation in a c-K-*ras* oncogene isolated by transfection analysis of DNA from liver tumors of winter flounder (*Pseudopleuronectes americanus*). They observed a single G→T transition in the second base of the 12th codon. This same type of mutation has been reported in *ras* genes isolated from chemically induced tumors in rodents (Barbacid, 1987).

Work in this research project has focused on the identification of transforming genes from chemically induced tumors in the Japanese medaka. In a previous report (Van Beneden et al., 1988), the highly transforming putative oncogene identified by transfection analysis and nude mouse assays was described. The fish tumor DNA used in these assays was isolated from a DEN-induced cholangiocarcinoma in medaka. In this report, progress in cloning and identification of this presumptive oncogene is presented.

MATERIALS AND METHODS

Transfection Procedure

DNA was isolated from foci from primary and secondary transfected cells by a modified Hirt extraction procedure. In order to identify transforming sequences, this DNA was examined by transfection assay. A stock of NIH 3T3 cells (490 N3T) was maintained at levels below confluency in Dulbecco-modified Eagle's medium supplemented with 10% fetal calf serum. To each plate of 3×10^5 NIH 3T3 cells, 25 μg of fish genomic DNA was cotransfected with 2 μg of a neomycin-resistant plasmid (pSV_2neo) in the presence of calcium phosphate (Graham and van der Eb, 1973). A total of four plates (100 μg fish DNA) of each sample was tested, which was expected to provide one genomic equivalent of DNA. Cells were grown in the presence of geneticin (G418) for 2 weeks. Drug-resistant colonies were selected, harvested by trypsinization, and replated (in the absence of G418) in a standard focus assay, in a colony selection assay, and/or injected into athymic mice (Blair et al., 1982).

To test the efficiency of the transfections, parallel secondary transfections were performed using DNA isolated from foci from the primary cotransfection, but without the addition of more pSV_2neo. Foci were picked and expanded, and DNA was isolated as described previously (Van Beneden et al., 1988). DNA digests from these foci were run on Southern blots and hybridized to radiolabeled pSV_2neo. Tertiary transfections were also done using DNA from these secondary foci, again without the addition of more pSV_2neo plasmid.

Southern Blot Analysis

Southern blots were prepared using DNA isolated from transfected cells (Southern, 1975). These were hybridized under conditions of high stringency (50% formamide, 5 x SSC, at 42°C) to either pSV_2neo or high-molecular-weight fish genomic DNA, which had been labeled with ^{32}P by the random primer method (Life Technologies, Inc.).

Preparation of Libraries

Two libraries were prepared. For the first, *Sau* 3A I partial digests of DNA from foci of tertiary transfectants (TR14-6Aa) were size selected on NaCl gradients and ligated into the *Bam*H I site of λ DASH (Stratagene). For the second, DNA from tertiary transfectant foci (TR14-6Aa) was digested with *Bam*H I, run on a 0.4% agarose gel, and fragments corresponding to 17-20 Kb were cut out, electroeluted, treated with bacterial alkaline phosphatase, and ligated into the *Bam*H I site of EMBL3 (Stratagene). Recombinant clones were packaged in Gigapack Gold according to the manufacturer's directions (Stratagene).

Screening of Libraries

The libraries were screened using P2392 host cells. Plates were transferred to nitrocellulose and hybridized to a pSV_2neo probe under high stringency conditions. Positive plaques from a tertiary screening cycle were isolated. Lysates prepared from these plaques were used in mini-prep DNA preparation. DNA was digested with *Sal* I to release insert, run on 0.8% agarose gels, and the Southern blots hybridized to radiolabeled pSV_2neo.

RESULTS

The efficiency of transformation was determined by secondary transfections using DNA isolated from drug-selected primary transfects. As shown in Table 1, on the average, 80 foci/plate (3.2 foci/μg DNA) were observed in plates that had been transfected with DNA isolated from primary foci. These primary foci had been produced by DNA isolated from

DEN-induced cholangiocarcinoma. No foci were observed on control plates (TR10-1 through TR10-4). These had been transfected with DNA isolated from primary transfectants that had been transfected with DNA from normal medaka livers. No foci were observed in plates transfected with calf thymus DNA.

Table 1. Transfection Efficiency: TR-10 Secondary Transfection (no additional plasmid)

DNA Source	Plate Number	Foci/Plate*
TR8-1A 3	TR10-1	0
(untreated medaka	TR10-2	0
controls)	TR10-3	0
	TR10-4	0
TR8-13Blg	TR10-5	78
(DEN-induced	TR10-6	74
cholangiocarcinoma)	TR10-7	100
	TR10-8	84
Calf thymus DNA	TR10-9	0

*25 μg of DNA was transfected per plate.

Table 2 shows the number of foci present when DNA isolated from cells of the secondary foci described above was transfected in a third cycle into NIH 3T3 cells, again with no additional plasmid added. Approximately 20 foci/plate were observed.

Southern blots of DNA digests of secondary and tertiary transfectants when hybridized to pSV_2neo showed distinct bands, homologous to the plasmid (data not shown). Only DNA from cells that had been transfected with fish tumor DNA had pSV_2neo-specific sequences. Thus pSV_2neo appeared to have integrated into the host genome near the same integration site as the fish transforming gene.

The libraries have been screened through three cycles. Twelve possible positive clones have been isolated, DNA has been prepared and digested with *Sal* I, and Southern blots have been prepared and hybridized to ^{32}P-pSV_2neo.

Table 2. Transfection Efficiency: TR-14 Tertiary Transfection
(no additional plasmid)

DNA Source	Plate Number	Foci/Plate*
TR10-4B	TR14-1	0
(untreated medaka	TR14-2	0
controls)	TR14-3	0
	TR14-4	0
TR10-6Ba	TR14-5	27
(DEN-induced	TR14-6	18
cholangiocarcinoma)	TR14-7	20
	TR14-8	16
TR10-8Ab	TR14-9	25
(DEN-induced	TR14-10	32
cholangiocarcinoma)	TR14-11	21
	TR14-12	22
Calf thymus DNA	TR14-13	0
	TR14-14	0

*25 μg of DNA was transfected per plate.

DISCUSSION

In a previous paper (Van Beneden et al., 1988) it was demonstrated that DNA isolated from medaka tumors was able to transform NIH 3T3 cells in standard transfection assays. The highest degree of transformation was elicited by DNA isolated from a DEN-induced cholangiocarcinoma. The researchers were unable to demonstrate homology of this presumptive fish oncogene to any known oncogene sequence by Southern blot analysis. This is consistent with results of studies by Stowers et al. (1988). They treated B6C3F mice and Fischer 344 rats with DEN, isolated DNA, and in similar transfection studies, looked for the presence of activated oncogenes. Their results suggest that DEN-induced rat and mouse liver carcinogenesis may involve genetic targets other than or in addition to the *ras* genes.

Recent secondary and tertiary transfection studies in the absence of additional pSV_2neo gave high efficiencies of transfection, equal to or greater than those reported for mammalian systems. These experiments showed that pSV_2neo sequences have apparently integrated into the host cell genome near the fish transforming sequence. This is now used as a marker to search for the fish gene. Screening of the libraries is currently in progress.

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NEOPLASIA IN FISH: TUMOR AND MECHANISM STUDIES IN TROUT

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INTRODUCTION

The long-range goals of this project were to understand the significance to human health of epizootics of neoplasia in feral fish populations and to expand basic knowledge of the comparative aspects of neoplasia in fish and mammals. Although suspect etiologic agents such as benzo(a)pyrene (BP), dibenz(a,h)anthracene (DBA), and carbazole (CBZ) have been identified in some epizootic locales, causal relationships have not been established. Definitive associations will require not only direct demonstration of carcinogenic potencies in fish, but also an understanding of the impact on tumor response of confounding variables including: water quality and temperature, nutritional status and growth rate, life stage at risk, genetic variation, population age structure, migration habits, and presence of tumor modulators as well as genotoxins. Also lacking has been an understanding of the basic molecular biology of carcinogenesis in fish compared to mammals. Although rainbow trout seldom inhabit polluted environments where fish tumor epizootics occur, they have received over 20 years of study as a tumor model and thus were among the few laboratory fish tumor models with sufficient development to address many of these needs.

It was proposed to use this model with the following specific aims: (1) conduct tumor studies to establish or confirm the carcinogenicities of BP, DBA, CBZ, and the model carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) in trout by selected exposure routes, and determine the impact on tumor response of potential modulating variables, including temperature, nutritional status, selected dietary cofactors (DDT, CBZ, phthalates, Aroclor 1254), and maternally accumulated embryonic Aroclor 1254; (2) conduct mechanism studies on procarcinogen pharmacokinetics, cellular metabolism, DNA adduct formation and repair, and the effects of the above modulating influences on these processes; (3) and further characterize fundamental aspects of tumor progression in trout, including development and progression of preneoplastic foci in liver and the transplantability of various tumor types into syngeneic hosts; and (4) investigate the possible involvement of oncogenes and tumor-transforming genes in trout neoplasia through the isolation and characterization of selected c-*onc* genes,

determination of their expression and multiplicity in normal and tumor tissue, and development of cell culture systems for oncogenic transfection assay.

During the past grant year, experiments have been conducted on each of the specific aims. Results on these experiments will be presented consecutively by specific aim.

SPECIFIC AIM 1: CARCINOGENICITY OF POLYNUCLEAR AROMATIC HYDROCARBONS

After demonstrating the carcinogenicity of DMBA to rainbow trout livers, stomachs, kidneys, and swim bladders by both embryo and fry static water immersion exposures, research was conducted to investigate the effects of single environmental co-contaminants on the carcinogenicity of this model polynuclear aromatic hydrocarbon (PAH). Both inhibitory (exposure to dietary contaminants prior to static water DMBA fry exposure) and promotional (dietary exposure after embryo initiation by DMBA) protocols were used. Two doses each of DDT, Aroclor 1254 (a PCB mixture), diethylhexylphthalate (DEHP), and CBZ were used in both protocols. The protocols are graphically presented in Figure 1. Embryo exposure was to 5 ppm DMBA for 3 hours, while fry exposure consisted of three 20-hour exposures to 1 ppm solution at weekly intervals. Each group was housed in a 3-foot circular fiberglass tank supplied with well water at 3 gpm and a 12:12 light:dark cycle. Necropsies occurred at 9 months postinitiation. Liver, stomach, kidney, and swim-bladder tissues were preserved in Bouin's solution for standard histological processing and light micropsy.

Results of the fry and embryo experiments are presented in Tables 1 and 2, respectively. Table 1 reveals that both doses of DEHP and DDT caused significant inhibition of DMBA liver carcinogenesis, while the high PCB dose enhanced the response. Stomach and swim-bladder tumor incidences were not significantly affected by the dietary pre-exposure. Post-embryonic exposure to these diets resulted in significant enhancement of the positive control value in all cases. Only liver tumors were initiated by embryo exposure to DMBA at this dose and exposure time. Thus, these experiments show that combined exposures to chemicals can enhance as well as inhibit tumor incidence. The most significant interaction in these combination exposures was that between PCB and DMBA. In both cases, high PCB doses enhanced the response to DMBA. Because PCBs and PAHs are frequently found together in polluted environments, the possible increase in carcinogenic risk from their combined effects is highly likely.

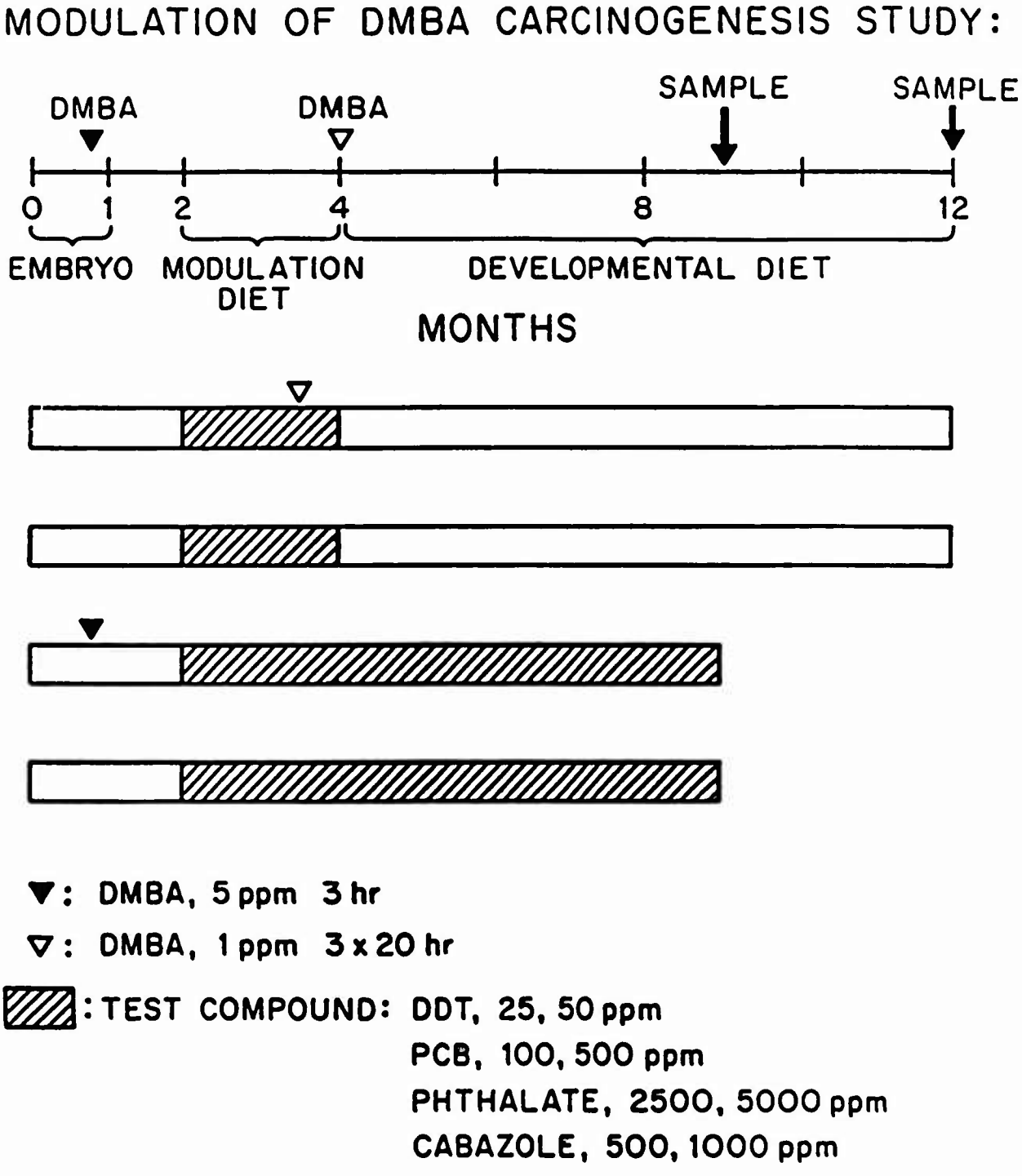

Figure 1. Protocols for DMBA carcinogenesis study.

Table 1. Effects of Contaminant Pre-exposure on DMBA Carcinogenesis in Rainbow Trout Fry

Pre-Initiation Diet (8 wks.)	DMBA Exposure	9-Month Hepatic Tumor Incidence	%	P Value
2,500 ppm DEHP	1 ppm/20 hr. × 3 (weekly)	43/184	23.4	<0.0001
5,000 ppm DEHP	" "	48/173	27.8	0.0005
100 ppm PCB	" "	60/147	40.8	0.3760
500 ppm PCB	" "	101/186	54.3	0.0078
12.5 ppm DDT	" "	54/186	29.0	0.0012
25 ppm DDT	" "	55/174	31.6	0.0085
500 ppm CBZ	" "	79/189	41.8	0.4433
1,000 ppm CBZ	" "	89/189	47.1	0.1989
OTD	" "	144/336	42.9	

Table 2. Effects of Continuous Contaminant Exposure Following Embryo Exposure to DMBA

Embryo Treatment	Diet Composition	9-Month Hepatic Tumor Incidence	%	P Value
5 ppm DMBA/3 hrs.	2,500 ppm DEHP	45/144	31.3	0.0008
" "	5,500 ppm DEHP	52/152	34.2	<0.0001
" "	100 ppm PCB	52/173	30.1	0.0010
" "	500 ppm PCB	66/172	38.4	<0.0001
" "	12.5 ppm DDT	49/129	38.0	<0.0001
" "	25 ppm DDT	46/147	31.3	0.0007
" "	500 ppm CBZ	54/129	41.9	<0.0001
" "	1,000 ppm CBZ	43/134	32.1	0.0005
" "	OTD	39/235	16.6	
Sham control	5,000 ppm DEHP	0/132	0	
" "	500 ppm PCB	1/141	0.7	
" "	25 ppm DDT	2/136	1.5	
" "	1,000 ppm CBZ	1/142	0.7	

SPECIFIC AIM 2: METABOLISM STUDIES ON PAHs

The thrust of the research for this specific aim was to investigate the uptake, metabolism, and DNA binding of DMBA in rainbow trout embryos. Samples of 200 trout embryos were incubated at 12°C in the dark in 100 ml well water containing 1 or 5 ppm [^{3}H]DMBA (500 μCi total radioactivity per 100 ml exposure solution). Aeration was provided, and incubation periods ranged from 0 to 24 hours. After treatment, embryos were rinsed with ice-cold water, frozen in liquid N_2, and stored at -70°C until analysis. A separate group of embryos was exposed to 5 ppm [^{3}H]DMBA for 24 hours and then transferred to fresh DMBA-free water, in which the embryos were held for an additional 24 hours before removal for analysis. Aliquots of the exposure solutions also were collected at various times for analysis.

The amount of radioactivity in treated embryos was determined at various times of exposure to measure the absorption and loss of DMBA. Whole embryos (three to five pools of three eggs per time point) were digested in NCS tissue solubilizer (Amersham Corp.). The digests were neutralized with acetic acid, suspended in OCS scintillator solution (Amersham Corp.), and counted in a Beckman LS7500 liquid scintillation counter. DMBA was absorbed rapidly by trout eggs during the 24-hour 5 ppm DMBA exposure, reaching 77% of the maximum egg DMBA level 2 hours after the start of exposure. No significant change in egg DMBA level occurred between 4 and 24 hours of DMBA exposure, but a 22% decrease in egg DMBA level was found 24 hours after transfer of eggs from the DMBA solution to fresh water. Approximately 13% of the DMBA in the 5-ppm bath was found to be associated with the 200 eggs treated in 100 ml H_2O after the 24-hour DMBA exposure.

Embryo homogenates were extracted twice with ethyl acetate/acetone (2:1), and the distribution of radioactivity between the organic and aqueous phases was determined by liquid scintillation counting of aliquots of each phase. Exposure solution samples similarly were extracted and analyzed. Analysis of the distribution of the egg-associated radioactivity into organic-soluble, aqueous-soluble, and protein-bound fractions revealed that while the percentage of organic-soluble radioactivity decreased over the 24-hour exposure, the percentage of the aqueous-soluble and protein-bound radioactivity increased over the same period. After transfer of the eggs from the DMBA bath to fresh water, the percentage of organic-soluble radioactivity continued to decline such that 24 hours after the transfer to fresh water, the aqueous-soluble and protein-bound fractions accounted for 46% of the total radioactivity. A comparison of the nonorganic fractions showed that the percentage of aqueous-soluble radioactivity was significantly higher than that of protein-bound radioactivity at all time points examined except at 2 hours, when no significant difference was found between the two nonorganic fractions. The highest level of covalently bound radioactivity,

8.8±0.8 pmol DMBA equivalent per mg protein, was found 24 hours after transfer of eggs to fresh water.

The aqueous phase of the embryo extractions was examined for the presence of glucuronide and sulfate conjugates of DMBA. Aliquots of the aqueous phase were filtered (0.45 μm pore size), bubbled gently with N_2 to remove residual acetone, and then combined with an equal volume of 0.2 M sodium acetate buffer (pH 5.0), or buffer containing β-glucuronidase (4000 Fishman units/ml) or arylsulfatase (20 units/ml, plus 40 mM D-saccharic acid 1.4-lactone). Enzymatic hydrolysis was conducted at 37°C for 4 hours. Released DMBA metabolites were extracted with ethyl acetate/acetone, and the distribution of radioactivity between the organic and aqueous phases was determined as described above. A significant amount of the aqueous-soluble radioactivity was hydrolyzed to organic-soluble material by β-glucuronidase treatment, but none of the aqueous-soluble radioactivity was released by arylsulfatase treatment under the present experimental conditions. Accumulation of β-glucuronidase releasable material was apparently proportional to the duration of DMBA exposure between 0 and 24 hours. No further increase in β-glucuronidase releasable material was found in the eggs 24 hours after transfer of eggs to fresh water. Although the amount of glucuronidase-releasable material increased with the time of DMBA exposure, the percentage of the total aqueous-soluble radioactivity that was released by glucuronidase treatment remained in the range of 27% (2 hours) to 20% (24 hours).

Primary metabolites of DMBA in the ethyl acetate fraction of the embryo extractions were analyzed. The ethyl acetate (3 pools of 10 embryos per time point) was dried over anhydrous sodium sulfate and then evaporated under a gentle stream of N_2. The residue was reconstituted in methanol and filtered through a 0.45-μm pore size fluoropolymer filter before analysis by reverse-phase HPLC. Metabolites were identified tentatively by coelution with reference standards. The major identified metabolites found in trout eggs at all time points examined were 12-OHMe-7-MeBA and DMBA-3,4-diol, reaching maximum levels of 44 and 94 pmol/egg, respectively, at 12 hours. The pattern of change in concentration over time for the two major metabolites, in which an increase during the first 12 hours of exposure was followed by a decline at later time points, also was found for the minor ethyl acetate extractable metabolites including 7,12-diOHMeBA, DMBA-5,6-diol, 7-OHMe-12MeBA, and DMBA phenols.

DNA from treated embryos was isolated for analysis of covalently bound radioactivity. Egg shells were cut open, embryos removed and rinsed free of yolk, and suspended in ice-cold Tris buffer. Embryos were lysed by vigorous mixing in an equal volume lysis buffer, and DNA was isolated from lysates of pools of 50 embryos each by phenolic extraction. The final

ethanol-precipitated DNA was washed three times each in ethanol, ethanol/ether (1:1), and ether to remove any noncovalently bound radioactivity. Purified DNA samples were dissolved in 2 ml water, and aliquots were counted for radioactivity and analyzed for DNA concentration by fluorometry. Significant DNA adduction was detected at 24 hours, and the level of adduction increased with time of DMBA exposure. The time-dependent increase in covalent binding continued after transfer of trout eggs to fresh water.

SPECIFIC AIM 3: TUMOR PROGRESSION AND TRANSPLANTABILITY

Experiments relating to this specific aim focused on the early stages of hepatocarcinogenesis in rainbow trout. Specifically, the interrelationships among carcinogen (aflatoxin B_1; AFB1) metabolism, DNA binding, target cells, cytotoxicity, regeneration, and carcinogenicity were investigated.

The roles of metabolism in AFB1 cytotoxicity and cytotoxicity in AFB1 hepatocarcinogenesis in rainbow trout have been examined. Groups of rainbow trout fry were exposed to carcinogenic aqueous solutions of 0.05, 0.1, 0.25, or 0.5 ppm [^{3}H]-AFB1 for 30 minutes. Another group of fry was fed 500 ppm β-naphthoflavone (BNF) for 1 week before exposure to 0.5 ppm AFB1 for 30 minutes. Subsamples of fish were killed 24 hours and 2 weeks later for DNA binding and histopathological analysis, respectively. Results indicated a linear dose response in both DNA binding and cytotoxicity. BNF treatment resulted in a decrease in both DNA binding and cytotoxicity. These results suggest that cytotoxicity, in common with carcinogenicity, is dependent on metabolism of AFB1 to the electrophilic 8,9-epoxide that can react covalently with cellular macromolecules, and that cytotoxicity contributes to, but is not required for, hepatocarcinogenesis. In a separate experiment, groups of fry were exposed to 0 or 0.5 ppm AFB1 for 30 minutes and subsamples of fish were given [^{3}H]-thymidine ([^{3}H]-TdR) i.p. at a single dose of 5 μCi/g body weight at 0, 1, 3, 7, and 14 days following carcinogen exposure, 24 hours prior to sacrifice. Autoradiograms showed intense radioactivity in presumptive oval cells, which were seen at 14 days after carcinogen exposure, but there was no labeling in degenerate, necrotic hepatocytes. These results suggest that oval cells are responsible for liver regeneration.

SPECIFIC AIM 4

Part of a trout *ras* gene has been isolated using the polymerase chain reaction (PCR) technique with various consensus *ras* primers. Previously reported PCR products of 111 bps and 216 bps are now believed to be carryover products from plasmid controls used in the

laboratory. However, the 800-bp product from a PCR using trout genomic DNA probed positively with a human H-*ras* probe and has been cloned into the Sma I site of pUC 10. Five clones have been mapped with 20 different restriction enzymes. A partial restriction map is shown in Figure 2. The five clones were sequenced using a modified TaqTrack (Promega) system and were shown to have a unique *ras* sequence in comparison to other vertebrate *ras* sequences.

One clone (RT-8) contained a consensus sequence of the five clones and encompassed the first exon, the first intron, and part of the second exon. The sequence of the exon portions of the insert is shown in Figure 3 with a comparison to the human H-*ras* gene. The homology of RT-8 to other known *ras* sequences (after exclusion of the primer regions) is 90.5% mammalian H-*ras*, 84.4% mammalian K-*ras*, and 89.0% goldfish *ras*. Southern transfers of trout genomic DNA digested with several restriction enzymes have been probed with the RT-8 insert and show unique bands undetected with human *ras* probes. The known trout *ras* sequence is being used with cDNA and several genomic libraries to isolate the entire trout *ras* gene. Primers have been synthesized that are specific for the trout sequence to isolate PCR products for analysis of the codon 12 and codon 61 regions in trout tumor DNA obtained from various organs and initiated with various carcinogens. Part of a second trout *ras* gene was identified and isolated using primers specific for the rat K-*ras* first exon in the PCR technique. The 90-bp PCR product has been cloned and named RT-11. The sequence of RT-11 is shown in Figure 4 with a comparison to several other vertebrate *ras* sequences. The homology of RT-11 to other known *ras* sequences (again with the exclusion of primer regions) is 84% mammalian H-*ras*, 78% mammalian K-*ras*, 76% goldfish *ras*, and 80% RT-8. Previously mentioned studies involving RT-8 are in progress with RT-11.

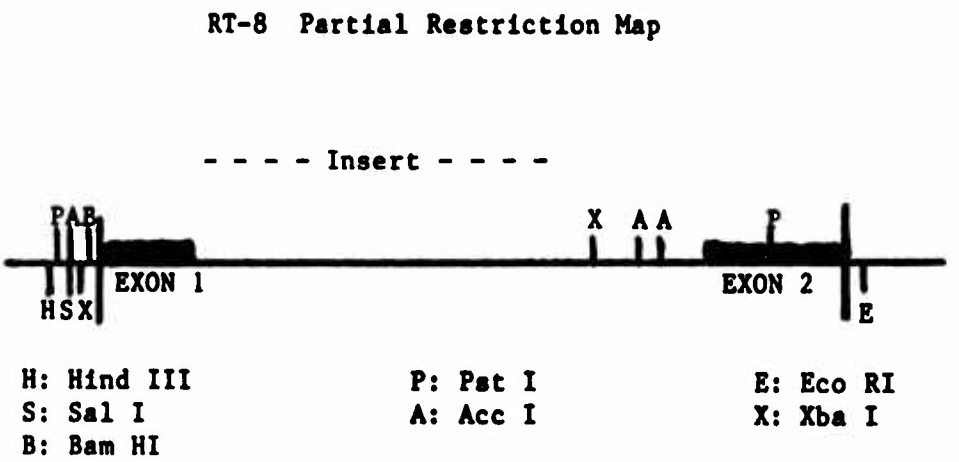

Figure 2. RT-8 partial restriction map.

ras SEQUENCE COMPARISON
Human H-*ras* and RT-8 Clone
EXONS 1 & 2

EXON 1

```
                10        20        30        40        50        60
Human H   ATGACGGAATATAAGCTGGTCCTGGTGGGCGCCGGCGGTGTGGGCAAGAGTGCGCTGACC
RT-8      ----------------T----------G--A--A-------------C-----C---

                70        80        90       100       110
Human H   ATCCAGCTGATCCAGAACCATTTTGTGGACGAATACGACCCCACTATAGAG
RT-8      --------C--T--------C--------T-----T--------C--C---
```

EXON 2

```
               120       130       140       150       160       170
Human H   GATTCCTACCGGAAGCAGGTGGTCATTGATGGGGAGACGTGCCTGTTGGACATCCTGGAT
RT-8      --C--G---A------------G-------------A--T---C------------C

               180       190       200       210       220       230
Human H   ACCGCCGGCCAGGAGGAGTACAGCGCCATGCGGGACCAGTACATGCGCACCGGGGAGGGC
RT-8      --T--A--T--------------------A-------A--------A-G----------

               240       250       260       270       280       290
Human H   TTCCTGTGTGTGTTTGCCATCAACAACACCAAGTCTTTTGAGGACATCCACCAGTACAGG
RT-8      -----C (End of insert)
```

Figure 3. A comparison of the RT-8 clone *ras* sequence and the human H-*ras* sequence.

ras SEQUENCE COMPARISON
RT-11 Clone and Others
EXON 1

EXON 1

```
                10         20         30         40         50         60
Human H  -----G--------G--G--G--G--G--C--C--C--T--G-----------GC----C
RT-8     -----G--------G-----G--G--G--G--A--A--T--G--------C--GC-C--C
Human K  ATGACTGAATATAAACTTGTTGTAGTTGGAGCTGGTGGCGTAGGCAAGAGTGCCTTGACG
RT-11    --------G-------------G--G--T-----A--T--T--G-----------A--T
Rat K    --------G-----------------------------------------------------
Mouse K  --------G-----------------------------------------C---------

                70         80         90        100        110
Human H  --C-----G--C-----C----------------C--C--C--T------
RT-8     --C-----C--------C--C--------T--------C--C--C--C---
Human K  ATACAGCTAATTCAGAATCATTTTGTGGACGAATATGATCCAACAATAGAG
RT-11    --C-----C----------C--------T (End of insert)
Rat K    -------------------C--------T----------T--G------
Mouse K  -------------------C--------T--G--------T--G------
```

Figure 4. The RT-11 *ras* sequence compared to several other vertebrate *ras* sequences.

DOSE-RESPONSE RELATIONSHIPS AT LOW CARCINOGEN CONCENTRATIONS IN A SMALL FISH CARCINOGENESIS MODEL

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ABSTRACT

The Japanese medaka (*Oryzias latipes* Temmick and Schlegel) is proposed for use in a large-scale study designed to produce a statistically relevant 1.0% (ED_{01}) and 0.1% (ED_{001}) N-nitrosodiethylamine [diethylnitrosamine (DEN); CAS 55-18-5] tumorigenic response and to determine the shape of the dose-response curve from the ED_{001} to the ED_{01} response. Endpoints of the study include hepatic tumorigenesis as persistent altered foci, hepatocellular adenoma and hepatocellular carcinoma, mortality, cause of death, toxic and adaptive change relative to tumorigenesis, and extrahepatic carcinogenesis. Preliminary investigations will occupy approximately 18 months of the project period and will involve: (1) establishment of a steering committee; (2) increasing medaka production; (3) investigation of defined or semi-defined diets; (4) characterization of DEN-induced tumor progression; (5) evaluation of the general shape of the dose-response curve from 1 to 10% tumorigenic incidence; (6) investigation of age at initial exposure relative to tumorigenesis and dosimetry; (7) refinement of analytical techniques; (8) confirmation of histopathological protocols; (9) finalization of statistical design; (10) design of dosimetry and/or pharmacokinetic studies; and (11) examination of systems for collecting, processing, storing, and retrieving large amounts of data. The definitive test will take an additional 18 months, approximately, and will involve about five replicate flow-through exposures, each involving about 7,500 medaka and 10 DEN concentrations. Following approximately 30 days' exposure, fish will be maintained in DEN-free dilution water until histopathological examination at 6 months postinitial exposure.

INTRODUCTION

Because most carcinogen bioassays that utilize rodent models identify risk at the 10-15% incidence levels, risk from potentially carcinogenic chemicals at incidence levels at 1% and below must be extrapolated (Krewski et al., 1989). Risk assessment studies, such as the ED_{01} study (Staffa and Mehlman, 1979), that target low-dose responses in mice require tens of

thousands of specimens and are compromised by difficulties in achieving uniform dosing and grow-out environments, by control incidences of neoplasms approaching those projected for low-dose exposure, and by cost. Because such studies have not provided data to adequately estimate risk, more information about the low end of the dose-response curve is needed.

MATERIALS AND METHODS

Fish Species

The Japanese medaka will be utilized in these studies. The medaka has been extensively used for embryological studies because of the regularity of its egg production and the clarity of its chorion and for physiological studies (Kirchen and West, 1976). Medaka have developed neoplastic lesions after being exposed to a wide variety of carcinogens, including nitroso compounds (DEN; N-methyl-N'-nitro-N-nitrosoguanidine), an azo compound (aminoazotoluene), a strong alkylating compound (methylazoxymethanol acetate), polynuclear aromatic compounds (benzo[a]pyrene; 7,12-dimethylbenzanthracene), and several mycotoxins (aflatoxin B_1; aflatoxin G_1; sterigmatocystin) (Couch and Harshbarger, 1985; Hatanaka et al., 1982; Hawkins et al., 1990; Metcalfe, 1989). Experimentation in the Gulf Coast Research Laboratory has demonstrated that, compared with other small fish species including guppy, sheepshead minnow, Gulf killifish, inland silverside, rivulus, and fathead minnow, the medaka: (1) develops hepatic tumors as rapidly or more rapidly than the other species, (2) exhibits as high or higher incidences of neoplasms as the other species, (3) provides extrahepatic tumorigenic responses not found in most of the other fish tested, and (4) is the most practical to rear in large numbers. Medaka are easily produced and maintained under laboratory conditions, can be readily manipulated to produce large numbers of similarly aged fry, and are easily housed for extended periods under grow-out conditions required for tumor development. Of particular interest to this investigation is the near absence of spontaneous tumors in the liver, the major target organ in this study, in control medaka through 6 months, the anticipated observational period in this study.

Test Chemical

The test chemical in this study will be DEN. DEN meets the selection criteria identified for a chemical carcinogen as promulgated by the National Center for Toxicological Research in its "Innovations in Cancer Risk Assessment (ED_{01} Study)" (Staffa and Mehlman, 1979). DEN, a known carcinogen in the model organism, is available in pure form, produces characteristic tumors in medaka, and is stable in the exposure medium. It is easily quantified

chemically, represents a class of chemicals, and is acceptable to the test animal. Additionally, considerable comparative data regarding DEN effects in rodents are available.

Exposure Mechanism

With the exception of range-finding acute bioassays, most preliminary exposures and the definitive study will be flow-through. Flow-through exposure provides stability of test chemical concentration as well as removal of potentially toxic, carcinogenic, or synergistic metabolites; maximizes water quality; and is generally accepted as the method of choice for chronic and subchronic toxicological evaluations. The definitive test is projected as a 14- to 28-day exposure (for each of about five temporal replications).

Histopathological Procedures

The histological procedures are designed to allow examination of large numbers of specimens and to survey most internal organs of each individual specimen. In the preliminary evaluations, whole adult fish will be processed. In the definitive test, only livers may be processed. If, however, preliminary studies show that DEN induces a considerable number of extrahepatic tumors within a 5- to 6-month period, the approach will be modified.

PRELIMINARY EVALUATIONS

During the initial 18 months of the project, preliminary investigations will be conducted to determine or clarify the following: (1) the ability to produce sufficient numbers of medaka fry without incorporation of methylene blue as a fungicide in the embryo-hatching solution, (2) general health and tumorigenic response in medaka fed exclusively a defined or semi-defined diet, (3) the progression of DEN-induced tumors in the medaka, (4) the most appropriate age of medaka for exposure, (5) the general shape of the DEN/medaka tumorigenic dose-response curve from an incidence of about 10% to one approaching 1%, (6) the water chemistry of DEN, (7) the appropriateness of current histopathological protocols, (8) the statistical validity of the definitive test design, and (9) DEN dosimetry and pharmacokinetics.

Items 1 to 6 will be assessed simultaneously, probably in sequential assays differing in age of fish upon initiation of exposure. While there is general agreement that younger, 6- to 10-day-old fry are most sensitive, larger fish in the 30-day-old range, if equally or about as sensitive tumorigenically, would be better suited for dosimetric and pharmacokinetic studies. Medaka will be exposed to five or six measured DEN concentrations for approximately 4 weeks under flow-through conditions and transferred to grow-out for tumor development.

Diet during exposure and grow-out will be exclusively defined or semidefined. Approximately six sampling times will be incorporated into each bioassay to evaluate tumor progression, and whole fish will be sectioned to describe extrahepatic tumorigenic processes. While the number of fry at each DEN concentration will be insufficient to statistically measure tumorigenic responses below 1%, it will be possible to describe the dose-response relationships at the higher DEN concentrations. This curve will form the basis of the definitive study. These exposures will probably be of unbalanced design and will require approximately 4,000 fry per fish age.

In these studies, the number and distribution of the earliest definable DEN-initiated changes will be determined, and the progression of the lesions will be followed. These studies will enable the researchers to identify carcinogen-induced changes, resolve the boundary between preneoplasia and neoplasia, and determine appropriate sampling protocols to ensure accurate discovery and diagnosis of tumors and quantitation of the tumorigenic response.

The statistical aspects of the design of the preliminary and definitive tests will continue to be evaluated through consultation with Dr. Daniel Krewski, Health and Welfare Canada, Ottawa, and input and approval will be solicited from statisticians within the U.S. Environmental Protection Agency and other U.S. government agencies. A steering committee composed of representatives of the governmental agencies and industries involved and others will evaluate protocols submitted by the Gulf Coast Research Laboratory and aid in refinement of the definitive test. Aspects of DEN dosimetry and pharmacokinetics will be cooperatively investigated by the principal investigators and the steering committee to determine which specific tests are most appropriate to the study and who should perform them.

THE DEFINITIVE TEST

The objective of the definitive test is to identify statistically verifiable hepatocarcinogenic incidences below 1% and to determine the relationship of these observed responses to predicted responses derived mathematically by linear extrapolation from a 10% tumor incidence. As determined by Krewski, large numbers of test animals will be required for each DEN concentration and for the control. Based on current knowledge of the medaka model and the mathematical requirements for establishing low-dose responses, the definitive test would be designed as follows: If d represents the carcinogen dose required to elicit a 1% hepatocarcinogenic response in medaka, the doses required in the definitive assay will be 0.05 d, 0.1 d, 0.2 d, 0.5 d, 1.0 d, 2.0 d, 5 d, 10 d, 15 d, and 20 d. The 0.05 d, 0.1 d, and possibly the 0.2 d doses are necessary as minimal doses to bound the low-dose risk. The 0.2 d and 0.5 d

doses serve to detect nonlinearity in the response, and the 5 d, 10 d, 15 d, and 20 d concentrations are included to describe the dose-response curve between 1 and 10% and beyond the 10% response level. The high dose in the 5 d to 20 d range is tentative pending the results of the preliminary dose-response assay. Numbers of test fry at each dose level are listed in Table 1.

Table 1. Number of Test Fry per Dose Level

Dose	Number of Test Fry
0 (Control)	3,000*
0.05 d	6,000
0.1 d	6,000
0.2 d	6,000
0.5 d	3,000
1.0 d	3,000
2.0 d	3,000
5 d	300
10 d	300
15 d	300
20 d	300

*Control sample size arbitrarily set equal to that at 1.0 d. Control sample size may be increased to 6,000.

The test will probably be performed in five replicate exposures at quarterly intervals, with each exposure involving 5,500 to 7,000 fry. The actual carcinogen concentration utilized to produce a 1% hepatocarcinogenic response (i.e., dose d) will be estimated from results of the preliminary assessments. From the preliminary evaluations, the dose (d) required to induce a 1% hepatocarcinogenic response can only be estimated because of the relatively small number of medaka at each treatment level. The inclusion of 0.5 d and 2.0 d in the definitive treatment scheme, therefore, becomes very important because of the possible error in estimating d. Age of medaka fry will be either 6 to 10 or 30 days and will not vary by more than 3 days. Exposure temperature will be 27±1°C, and length of grow-out is expected to be 6 months.

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EVALUATION OF EPIGENETIC CARCINOGENS IN RAINBOW TROUT BY ASSESSING PEROXISOME PROLIFERATION POTENTIAL

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INTRODUCTION

This report presents a summary of the findings of the first-year research concerning the effects of two known rodent peroxisome proliferators (colfibrate and ciprofibrate) on the hepatic peroxisome proliferation in the rainbow trout. Results of the remaining five agents will be reported in a separate publication.

MATERIALS AND METHODS

Chemicals

Chemicals used in these studies were obtained from the following sources: clofibrate (Sigma, St. Louis, MO) and ciprofibrate (Sterling-Winthrop Research Institute, Rensselaer, NY). Sources for all other chemicals are provided elsewhere in the text.

Animals and Treatment

Rainbow trout, 1+ years in age with average body weight of 450 grams, were obtained from the Massachusetts Division of Fish and Wildlife and maintained in raceways at the Division's Sunderland Fish Hatchery. During experimentation, individual holding tanks were used to facilitate handling of fish and to group fish according to dosage. Groundwater was supplied to the tanks at a rate of approximately 113 liters/minute at temperatures ranging from 3°C to 12°C, depending on seasons of the year. Water temperature did not exceed ±2°C variation during any one exposure experiment. Water quality was determined by the Environmental Analysis Laboratory, University of Massachusetts.

Two hypolipidemic drugs were used in the study: clofibrate (ethyl-p-chlorophenoxyisobutyrate) and ciprofibrate (2-[4-(2.2-dichloro-cyclopropyl)phenoxy] 2-methyl propionic acid).

An initial range finding was conducted for each chemical to determine the maximum dose that did not result in behavioral or health effects such as abnormal swimming pattern, reduction of food consumption, or acquisition of fungus. Initially, a range of six doses was

chosen, based on the level administered in rodent studies. Chemicals were administered to three fish per dose via intraperitoneal (i.p.) injection every other day over a period of 2 weeks for clofibrate and ciprofibrate and every day over a period of 2 weeks for lactofen and DEHP. The highest dose that caused no behavioral or health effects was selected as the maximum experimental dose and used to extrapolate downward to two or three lower dosages.

A sample size for each chemical exposure was determined, based on the maximum number of trout that could be processed in the laboratory, because standard deviations of acyl-CoA oxidase activities in control and treatment groups from the range-finding study were high (approximately 30% of the mean values).

Prior to chemical exposure, trout were acclimated at least 3 days in individual holding tanks and then tagged with various colors by a tagging gun, according to the concentrations of exposure. For i.p. injection, trout were netted and put in plastic containers of 10 liters of water containing 100 ppm of tricain (3-aminobenzoic acid ethyl ester). A final solution containing 0.1 ml of the substance was injected, using a 26-gauge needle. Trout were anesthetized every day before injection.

Clofibrate was administered via i.p. injection to trout every other day over a period of up to 4 weeks at concentrations of 0, 15, 25, 50, and 75 mg/kg of body weight. Twenty fish were used in each concentration. Ten fish were sacrificed at the end of week 3 and week 4, respectively.

Ciprofibrate was administered via i.p. injection to fish every other day over a period of up to 3 weeks at concentrations of 0, 15, 25, and 35 mg/kg of body weight. Twenty fish were used in each concentration, and 10 fish per group were sacrificed at the end of weeks 2 and 3, respectively.

At the end of each exposure period, trout were sacrificed using tricain. Whole fish were weighed and the liver removed, weighed, and processed as described below. In all cases, test chemicals were dissolved in a saline-emulphor (10:1) mixture. Controls were injected with the saline-emulphor mixture only.

EXPERIMENTAL METHODOLOGY

Preparation of Light Mitochondrial Fraction

A light mitochondrial fraction containing peroxisomes was prepared according to Small et al. (1985). This procedure involved homogenation of 2.5 grams of liver tissue in four volumes of 10% sucrose containing 3 mM of imidazole (pH 7.4) (SI medium). The homogenate was centrifuged at 6,000 g for 10 minutes to remove connective tissues, intact cells, and nuclei. The supernatant was removed and stored in ice. Then, the remaining pellet was

resuspended with SI medium and recentrifuged as above. The combined supernatant was centrifuged at 30,000 g for 30 minutes at 6°C to sediment a pellet containing peroxisomes, mitochondria, and lysosomes. The pellet was resuspended in the SI medium for the enzyme assay, and protein concentration was determined using a Sigma protein assay kit based on Lowry et al. (1951).

Peroxisomal Acyl-CoA Oxidase Assay

The activity of peroxisomal acyl-CoA oxidase was measured according to Small et al. (1985). The assay is based on the measurement of oxidized DCF (2,7-dichlorofluorescin diacetate) by hydrogen peroxide, which is produced by peroxisomal beta-oxidation in the presence of the substrate, palmitoyl-CoA. Preliminary studies using six control fish indicated that the optimal assay temperature for trout was 30°C, based on the low standard deviations of peroxisomal enzyme activities. Thus, the assay was conducted in a cuvette at 30°C in a final volume of 2 ml. The assay mixture contained 38 μl of 0.05 mM leuco-DCF (prepared daily at 2.6 mM in one volume of N-N-dimethylformamide and nine volumes of 0.01 M NaOH, stored in a light-tight container under N2 gas), 200 μl of 40 mM aminotriazole, 0.16 mg of horseradish peroxidase, 20 μl of 2% triton-x, 1.682 ml of 11 mM potassium phosphate buffer (pH 7.4), and 20 μl of light mitochondrial fraction. This mixture was preincubated in a dark water bath at 30°C for 5 minutes. Increases in oxidized DCF levels were measured spectrophotometrically at 502 nM. The enzyme activity of fatty acyl-CoA oxidase was expressed as nanomoles (nM) of oxidized DCF per minute per gram of liver tissue.

Analysis of Polypeptide PPA-80 and Catalase Protein

PPA-80 (peroxisome proliferation-associated polypeptide protein; mol. wt. ~80,000) (Laemmli, 1970) was analyzed by SDS-PAGE as described below. The band of the catalase protein was identified by the immunoblot analysis, and the amount of protein was analyzed by SDS-PAGE. The quantities of both PPA-80 and catalase protein were determined by densitometric analysis.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Light mitochondrial fractions were suspended in 1% SDS containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein was determined with the Sigma protein kit (Sigma Chemical Co., St. Louis, MO). Samples were subjected to SDS-PAGE in 7.5% gels according to Laemmli (1970). Molecular mass marker proteins (Sigma) included ovalbumin (43,000), bovine serum albumin (66,000), phosphorylase b (97,000), β-galactosidase (116,000),

and myosin (205,000). The proteins in the gel were stained with Coomassie Brilliant Blue. The protein bands were scanned by a MicroScan 1000 gel-scanning system (Technology Resources, Inc., Nashville, TN). Densitometric results are reported in terms of percent protein present in the band of interest relative to total protein in the sample gel lane.

Purification of Peroxisomal Enoyl-CoA Hydratase

Peroxisomal enoyl-CoA hydratase was purified by the method of Osumi and Hashimoto (1979). The light mitochondrial fraction from 20 grams of trout liver was suspended in 10 mM potassium phosphate, pH 7.0, containing 2 mM 2-mercaptoethanol and 0.1% hexamethylphosphoric triamide (all buffers in this procedure contained these reagents) and applied on a phosphocellulose column (100 ml bed volume, equilibrated with 50 mM potassium phosphate, pH 7.0). The column was eluted with a linear gradient system (50-500 mM phosphate buffer in a total volume of 400 ml). The fractions with enoyl-CoA hydratase activities were pooled and treated with ammonium sulfate, and the protein precipitated between 200 and 400 grams/liter was dissolved in a minimal volume of 50 mM potassium phosphate, pH 7.0. The ammonium sulfate was removed by passage through a Sephadex G-25 column. The enzyme solution was diluted with an equal volume of cold water containing 2 mM 2-mercaptoethanol and 0.1% hexamethylphosphoric triamide. This was applied in a CM-cellulose column (25 ml bed volume, equilibrated with 25 mM potassium phosphate, pH 7.0), washed with 25 ml of 50 mM potassium phosphate, pH 7.0, and eluted with a linear gradient system of the buffer from 50 to 200 mM in a total volume of 300 ml. The active fractions of enoyl-CoA hydratase were pooled, and enzyme was precipitated by the addition of ammonium sulfate (350 grams/liter eluate).

Enzyme Assay for Peroxisomal Enoyl-CoA Hydratase

Heat-labile peroxisomal enoyl-CoA hydratase activity was measured by the method of Steinman and Hill (1975) in 0.3 M Tris-HCl, pH 7.4, containing 5 mM EDTA, 0.05 mg/ml ovalbumin, and 200 μM crotonyl-CoA (Sigma) as the substrate. The enzyme precipitations were diluted with 50 mM potassium phosphate, pH 7.0, and heated at 57°C for 5 minutes. The decrease in absorbance was recorded at 280 nM.

Immunoblot Analysis

The purified peroxisomal fractions and the purified enoyl-CoA hydratase were used for the immunochemical identifications of catalase and polypeptide PPA-80, respectively. Peroxisomes were isolated from the light mitochondrial fraction of trout hepatocytes using a

Nycodenz (Accurate Chemical and Scientific Research Corp., Westbury, NY) gradient according to Ghosh and Hajra (1986), and enoyl-CoA hydratase was purified as described above. Samples were subjected to SDS-PAGE as described above. All steps were carried out at room temperature, and all buffers contained 0.1 mg/ml nonfat dry milk (Carnation) unless otherwise stated Proteins were transferred to nitrocellulose paper (Schleicher and Schuell, Inc., Keene, NH) by a modification of the method of Towbin et al. (1979). The nitrocellulose paper was saturated with 50 mM Tris-HCl, 0.1 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.05% monolaurate polyoxyethylenesorbitan (TWEEN-20, Sigma), pH 7.25, containing 5 mg/ml nonfat dry milk for 2 hours, followed by incubation overnight at 4°C with a 1:1,000 dilution of rabbit anti-human catalase (Biodesign, Inc., Kennebunkport, ME) for catalase or rabbit antirat peroxisomal bifunctional enzyme (PBE) (Northwestern University, Chicago, IL) for PPA-80 in the Tris-HCl buffer. The nitrocellulose paper was then washed 3 x 30 minutes with the Tris-HCl buffer, incubated for 2 hours with a 1:1,000 dilution of alkaline phosphatase conjugated goat antirabbit IgG (Sigma) in the Tris-HCl buffer, and washed 3 x 30 minutes with the Tris-HCl buffer. For the color reaction, the nitrocellulose paper was soaked in a solution of 50 mM Tris, 150 mM NaCl, 50 mM $MgCl_2$, pH 9.5, containing 0.08 mg/ml 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) (Sigma) and 0.17 mg/ml nitroblue tetrazolium (NBT) chloride (Sigma). The reaction was terminated by washing with water.

Measurement of Body and Liver Weights

At the beginning and the end of each study period, body weights were measured by placing trout on a pan-balanced scale immediately after being taken from anesthetized water. The percent increase of body weight was calculated. The liver was taken from the trout by scissors and tweezers and the gallbladder was carefully removed by scissors at sacrifice. Then, the liver was immediately placed in a weigh boat on ice and weighed by a fine scale. Liver weights were expressed as grams of liver per 100 grams of total body weight.

Electron Microscopy

The fixation of trout liver tissue for electron microscopy began immediately upon the dissection of the fish. Liver tissue was analyzed from three fish from the high-dose and control groups of ciprofibrate-exposed fish (3 weeks). Liver tissue was prepared using the following procedure (Novikoff et al., 1973).

Approximately 10 mg of liver tissue was removed and placed in a solution of 0.2 M cacodylate, 2% glutaraldehyde, and 0.15 M sucrose, pH 7.4. The tissue was chopped with a

razor blade into 1×1×1-mm sections, transferred to 10-ml capped glass vials, and fixed for 3 hours at 4°C in 2 ml of the above-mentioned solution. The tissue was washed in cold buffer (0.2 M cacodylate, 0.3 M sucrose), rechopped with a razor blade, and stained with a solution of 0.2% 3,3-diaminobenzidine (DAB), 0.02% H_2O_2, 0.005 M KCN, and 0.1 M Tris-HCl, pH 9.0. The DAB stain is specific for the enzyme, catalase, within the peroxisome. Thus, the DAB staining allows one to distinguish the peroxisomes from other small, circular organelles in the cells. The tissue pieces were incubated in DAB for 30 minutes at 37°C. After incubation, the tissue pieces were washed with cold buffer and postfixed with 1% osmium tetroxide (Polyscience, Warrington, PA) for 1 hour at 4°C. The tissue was washed and dehydrated with a series of ethanol solutions (20, 50, 70, 95, and 100%) for 10 minutes per solution. Propylene oxide was gradually added with ethanol (50, 67, and 75% propylene oxide in ethanol), followed by 100% propylene oxide.

The tissue pieces were embedded in Epon 812, cut with a diamond knife (<0.1 μ thick section), and stained with uranyl acetate and lead citrate. The sections were viewed under a transmission electron microscope (Jeol 1005). Three blocks per animal were examined. Twenty photographs per block were taken at primary magnification of 5,000× and enlarged 2.6 times at printing to a final magnification of 13,000× for the convenience of analysis.

Statistical Analysis

The study design for the dose-response phase consisted of two independent variables (time and dose, or sex and dose) and five dependent variables (peroxisomal acyl-CoA oxidase, PPA-80 polypeptide, catalase, % increase of body weight, and liver weight). Only three dependent variables (peroxisomal enzyme activities, % increase of body weight, and liver weight) were used in the clofibrate study, A two-way analysis of variance (BMDP4V, Northwestern University, IL) was used in the multiple comparisons of dependent variables based on time and dose (clofibrate and ciprofibrate) and sex and dose (lactofen and DEHP). A p value < 0.05 was regarded as statistically significant. A p value $\geq$ 0.05 and $\leq$ 0.1 was considered marginally different from the respective control.

A trend analysis using a linear regression method was used to detect if there was a linear trend of each dependent variable over dose ($p = 0.05$). For a morphometric analysis, numerical density and volume density of the control and the treatment group were compared, using a two-tailed t-test ($p = 0.05$).

RESULTS

Clofibrate

Activity of Peroxisomal Acyl-CoA Oxidase (Table 1)

After the 3-week exposure, peroxisomal acyl-CoA oxidase activities in the 25 mg/kg and 75 mg/kg groups showed 28% ($p = 0.03$) and 34% ($p = 0.01$) increases over the control group, and there was a marginal difference ($p = 0.1$) between the 15 mg/kg group and the control group.

In contrast, there were no significant differences between the control and treatment groups after 4 weeks' exposure. While a marginally significant dose-dependent increase ($p = 0.08$) was observed after 3 weeks' exposure, there was no such trend observed after the 4-week exposure. Analysis for a time effect between weeks 3 and 4 showed significantly decreased enzyme activities of the 15 mg/kg ($p = 0.01$) and 75 mg/kg ($p = 0.03$) groups in week 4.

Percent of Body Weight Increase (Table 2)

No significant differences in body weight changes between the control and treatment groups were observed after the 3-week exposure. In contrast, there were significant decreases in the 15 mg/kg ($p = 0.0001$), 50 mg/kg ($p = 0.0008$), and 75 mg/kg ($p = 0.001$) groups after 4 weeks' exposure, as compared to the control group. The trend analysis did not show any significant dose-related change of body weights after the 3-week exposure, but did show a significant ($p = 0.003$) dose-dependent decrease after the 4-week exposure. A significant ($p = 0.01$) increase of body weight between weeks 3 and 4 was observed only in the control group.

Liver Weight (Table 2)

There was a liver weight decrease of 40% and 27% in the 25 mg/kg ($p = 0.02$) and 50 mg/kg ($p = 0.03$) groups, respectively, after the 3-week exposure, as compared to the control group. In addition, a marginal decrease was observed in the 15 mg/kg ($p = 0.09$) and 75 mg/kg ($p = 0.07$) groups after 3 weeks. A 28% decrease of liver weight ($p = 0.04$) was observed in the 15 mg/kg group after the 4-week exposure, and marginal decreases were found in the 50 mg/kg ($p = 0.07$) and 75 mg/kg ($p = 0.07$) groups. While data collected after the 3-week exposure showed a marginally significant dose-dependent decrease ($p = 0.06$) of liver weights, no significant trends of liver weight changes were observed in the week 4 data. No time-effect differences were seen between weeks 3 and 4 ($p > 0.05$).

Table 1. Effects of Clofibrate on Peroxisomal Acyl-CoA Oxidase Activities[a]

Time	Dose (mg/kg)[b] 0	15	25	50	75
Week 3	70.57 ±15.57	86.65[c] ±16.59	90.65[d] ±33.10	73.37 ±18.75	94.82[d] ±24.49
% Increase	0 (8)	23.3 (9)	29.0 (10)	4.2 (10)	34.0 (10)
Week 4	78.28 ±12.41	63.07 ±20.84	73.88 ±19.59	75.09 ± 8.90	74.23 ±14.03
% Increase	0 (9)	-19.4 (9)	-5.6 (10)	-0.4 (9)	-0.5 (9)

[a]Unit: DCF oxidized (nM)/min/g of liver tissue.
[b]Values are means ± S.D. Sample size in parentheses.
[c]Marginally different from respective control, $0.05 \leq p \leq 0.1$.
[d]Significantly different from respective control, $p < 0.05$.

Ciprofibrate

Peroxisomal Acyl-CoA Oxidase Activity (Table 3)

There were no significant differences between control and treatment groups for peroxisomal acyl-CoA oxidase activities following 2 weeks of exposure to ciprofibrate. In addition, no trend of difference over dose ranges was observed. However, after 3 weeks of exposure, enzyme activities in the high-dose (35 mg/kg) group showed a 78% increase (p = 0.0008) over the control group, and a marginal increase (p = 0.1) was observed in the mid-dose (25 mg/kg) group. In contrast to the 2-week results, a trend analysis of the 3-week data showed a significant dose-dependent increase of enzyme activities (p = 0.0005). Analysis for a time-effect difference between weeks 2 and 3 showed no significant difference in enzyme activity within each dose group.

PPA-80 (Table 4)

The immunoblot analysis of the purified enoyl-CoA hydratase showed an immunochemical reaction of anti-PBE antibody with a single protein band of molecular weight, 79,000. There were no significant differences between control and treatment groups

Table 2. Alterations of Body and Liver Weights After the Administration of Clofibrate[a]

Time	Dose (mg/kg)[b] 0	15	25	50	75
Body Weight (%) Increase[b]					
Week 3	3.49 ±12.55 (8)	-0.80 ±7.71 (9)	-1.22 ±12.70 (10)	-4.07 ±10.38 (10)	-3.41 ±8.87 (10)
Week 4	17.01 ±8.61 (9)	-1.73[d] ±5.29 (9)	8.34[c] ±12.11 (10)	0.43[d] ±4.40 (9)	-3.43[d] ±10.24 (9)
Liver Weight (g)[b]					
Week 3	1.96 ±0.59 (8)	1.60[c] ±0.31 (9)	1.40[d] ±0.32 (10)	1.54[d] ±0.25 (10)	1.60[c] ±0.53 (10)
Week 4	1.81 ±0.36 (9)	1.41[d] ±0.34 (9)	1.81 ±0.61 (10)	1.44[c] ±0.27 (9)	1.45[c] ±0.33 (9)

[a]Liver weights were expressed as gram of liver weight per 100 grams of body weight.
[b]Values are means ± S.D. Sample size in parentheses.
[c]Marginally significant from respective control, $0.05 \leq p \leq 0.1$.
[d]Significantly different from respective control, $p < 0.05$.

for PPA-80 content after 2 weeks of exposure, whereas there was a significant increase over controls after 3 weeks in the 25 mg/kg ($p = 0.01$) and 35 mg/kg groups ($p = 0.0001$). Increases in PPA-80 protein were 18% and 48% in the 25 mg/kg and 35 mg/kg groups, respectively. Trend analysis showed a significant dose-dependent increase for week 3 only ($p = 0.0005$). An analysis of time-effect difference showed a 36% increase of this protein ($p = 0.0001$) in the 35 mg/kg group in week 3, compared to the same group in week 2. No other time-related differences between weeks 2 and 3 were observed.

Relative Percent Protein of Catalase (Table 5)

The catalase band was immunochemically identified with rabbit antihuman antibody. There was only a marginal increase ($p = 0.08$) for catalase protein between the control and 35

Table 3. Effects of Ciprofibrate on Peroxisomal Acyl-CoA Oxidase Activities in Rainbow Trout[a]

Time	Dose (mg/kg)[b] 0	15	25	35
Week 2	66.57±25.10	58.40±18.18	65.55±22.87	75.80±13.81
% Increase	0	-12.2	-1.5	13.8
	(10)	(9)	(10)	(10)
Week 3	51.31±30.39	58.12±21.64	68.91±22.28	91.31±34.01[c]
% Increase	0	7.0	27.3	78.0
	(10)	(10)	(9)	(8)

[a]Unit: Nanomole DCF oxidized/min/g of liver tissue.
[b]Values are means ± S.D. Sample size in parentheses.
[c]Statistically significant from respective control, $p < 0.05$.

Table 4. Effects of Ciprofibrate on Relative Protein of PPA-80 in a Light Mitochondrial Fraction[a]

Time	Dose (mg/kg)[b] 0	15	25	35
Week 2	9.97±1.92	10.84±2.06	10.48±1.86	10.92±1.70
% Increase	0	8.7	5.1	9.5
	(10)	(9)	(10)	(10)
Week 3	10.04±0.84	10.65±1.45	11.88±0.66[b]	14.81±1.26[b]
% Increase	0	6.1	18.3	47.5
	(10)	(10)	(9)	(8)

[a]Values are means ± S.D. Sample size in parentheses.
[b]Significantly different from respective control, $p < 0.05$.

mg/kg groups after 2 weeks' exposure, while a significant increase was observed in the 15 mg/kg ($p = 0.03$) and 35 mg/kg ($p = 0.0001$) groups; a marginally significant increase ($p = 0.1$) was observed in the 25 mg/kg group after 3 weeks of exposure. The amount of catalase protein in week 3 was increased by 2.4- and 3.5-fold in the low- and high-dose groups, respectively.

Trend analysis for week 2 data showed only a marginally significant ($p = 0.06$) increase of catalase protein over dose. In contrast, significant ($p = 0.0002$) dose-dependent increases were observed after 3 weeks of exposure. There were no significant time-effect differences between weeks 2 and 3 in each dose group.

Percent of Body Weight Increase (Table 6)

No significant differences between control and treatment groups for percent body weight increase were found in weeks 2 and 3. There were no time-effect differences between weeks 2 and 3 in each dose group. Large variations among individuals in each dose group were observed in both periods of study. No statistically significant trend effect was shown in either period.

Liver Weight (Table 6)

There were no significant differences between control and treatment groups after 2 and 3 weeks of exposure. Only the high-dose group in week 3 showed a marginal ($p = 0.09$) increase over the control group. No trend for changes in liver weight was seen in either period. No time-effect differences were shown between weeks 2 and 3.

Morphometric Analysis (Table 7)

Because the high-dose (35 mg/kg) group of ciprofibrate after 3 weeks showed the highest increase (78%) of enzymatic activities over the control group, the high-dose group and its control group were chosen to be studied by morphometric analysis. Numerical densities and volume densities of hepatic peroxisomes were analyzed and compared. While numerical density showed no significant difference, volume density in the treatment group showed a 2.3-fold increase ($p = 0.01$) of peroxisomal volume over the control group. No increase of mitochondrial volume density was observed. Electron micrographs showed the absence of the crystalloid core in hepatic peroxisomes of rainbow trout with the mean length of 0.4 μm.

Table 5. Effects of Ciprofibrate on the Relative Percent Protein of Catalase in a Light Mitochondrial Fraction

Time	Dose (mg/kg)[a] 0	15	25	35
Week 2	3.26±2.49[a]	4.25±2.45	4.81±1.79	4.97±1.84[b]
% Increase	0	30.3	47.5	52.4
	(10)	(9)	(10)	(10)
Week 3	1.97±1.46	4.88±2.75[c]	3.61±1.95[b]	6.63±2.09[c]
% Increase	0	147.7	83.3	236.5
	(10)	(10)	(9)	(8)

[a]Values are means ± S.D. Sample size in parentheses.
[b]Marginally different from respective control, $0.05 \leq p \leq 0.01$.
[c]Significantly different from respective control, $p < 0.05$.

Table 6. Alteration of Body Liver Weights After Administration of Ciprofibrate

Time	Dose (mg/kg)[a] 0	15	25	35
Body Weight Increase (%)[a]				
Week 2	-6.72±8.95 (10)	-2.21±7.97 (9)	-4.06±4.22 (10)	-3.75±5.83 (10)
Week 3	0.99±8.27 (10)	-3.87±9.41 (10)	-3.32±10.69 (9)	-0.76±8.80 (8)
Liver Weight (g/100 g of body tissue)[a]				
Week 2	1.66±0.42 (10)	1.50±0.34 (9)	1.66±0.47 (10)	1.50±0.29 (10)
Week 3	1.50±0.24 (10)	1.65±0.31 (10)	1.53±0.37 (9)	1.81±0.53[b] (8)

[a]Values are means ± S.D. Sample size in parentheses.
[b]Marginally different from respective control, $0.05 \leq p \leq 0.01$.

Table 7. Peroxisomal and Mitochondrial Volume Densities of Ciprofibrate-Induced Peroxisome Proliferation in Liver of Rainbow Trout

	Peroxisomal	Mitochondrial
Control	1.67±0.02[a]	35.10±6.46
35 mg/kg	4.00±0.09[b]	33.40±8.92

[a]The values are expressed as percent of cytoplasmic volume ± S.D.
[b]Statistically significant from the control, $p < 0.05$.

DISCUSSION

Two hypolipidemic drugs (clofibrate and ciprofibrate) were used in the present study. Clofibrate is the most extensively studied hypolipidemic drug regarding its peroxisome proliferation capacity, and ciprofibrate is several times more potent and structurally related to clofibrate (Barber et al., 1987). The results showed a significant increase of acyl-CoA oxidase activities in rainbow trout after 3 weeks' exposure to clofibrate, but no increase after 4 weeks' exposure. This is contrary to the findings from the rodent studies, which show a sustained level of increase as long as the drug is administered (Reddy and Lalwani, 1983). It is speculated that the unhealthy condition of fish and/or leakage of drug from the injected area that was shown after 3 weeks of exposure might be the causes for the reduction of enzyme activities after 4 weeks' exposure. Still, adaptability of rainbow trout to this drug cannot be ruled out as a possible explanation.

While clofibrate causes hepatomegaly in rodents, which is believed to be associated with peroxisome proliferation (Reddy and Lalwani, 1983), it causes a decrease of liver weight in rainbow trout. It is suggested that potency and/or dosage of drug used in this study may not be high enough to induce liver weight increases, considering the level of enzymatic induction (34%). In mice, a twofold increase of peroxisomal oxidase resulted in only about a 30% increase of liver weight (Reddy and Lalwani, 1983).

A significant reduction of body weight after a 4-week exposure indicates the apparent toxicities in fish by this chemical. This may explain, in part, the reduction of enzyme activities after 4 weeks' exposure. Although a direct comparison with rodent response is very difficult due to the alternating injection scheme in the present study, it seems that the rainbow trout is more sensitive to this chemical, compared with rodents, which can easily tolerate up to 250 mg/kg via i.p. injection for a similar period of exposure (Yang and Leonard, 1988).

When the very potent peroxisome proliferator, ciprofibrate, was used in the study, the study period was reduced from 3-4 weeks to 2-3 weeks, due to the apparent toxicities and leakage of drug that were observed beyond the third week of clofibrate exposure. The results showed that ciprofibrate caused higher increases of acyl-CoA oxidase activities at a lower dose than clofibrate, indicating that ciprofibrate is a more potent peroxisome proliferator in rainbow trout. This relative ranking of the capacities to increase peroxisomal enzymes by these compounds is similar to that of rodents. It is interesting to note that values of this enzyme in untreated trout were in a similar range to those of untreated rats, which were measured with an identical assay protocol in the researchers' laboratory (Yang and Leonard, 1988).

While activities of catalase in rodent species are induced to a lesser degree by hypolipidemic drugs such as clofibrate and ciprofibrate than those of peroxisomal beta-oxidation enzymes (Reddy and Lalwani, 1983), the treatment of rainbow trout with ciprofibrate showed a higher degree of induction in catalase protein than in peroxisomal acyl-CoA oxidase. The level of induction measured by SDS-PAGE was similar to the maximum induction of catalase reported in rodent species (Reddy and Lalwani, 1983). If this phenomenon actually represents a cellular reaction to this compound and the mechanism of hepatocarcinogenesis by a peroxisome proliferator follows a theory of unbalanced hydrogen peroxide production in peroxisome, it may be suggested that rainbow trout is less vulnerable to hepatocarcinogenicity by this compound than rodent species. In any case, because all agents capable of inducing peroxisome proliferation in liver cells of rodents are able to increase the activities of liver catalase (Reddy and Lalwani, 1983), induction of catalase in rainbow trout is suggested to be evidence for peroxisome proliferation.

The results also demonstrated that ciprofibrate induced a significant dose-dependent increase of polypeptide PPA-80 in light mitochondrial fractions of trout liver. Because this protein is believed to be a major component of peroxisomes induced specifically by peroxisome proliferators and represents the entire fatty acid peroxisomal beta-oxidation enzyme system (Reddy et al., 1982), an increase of this protein is considered a strong indication of peroxisome proliferation in trout liver. In fact, the existence of polypeptide PPA-80 in liver of rainbow trout was first identified immunochemically in this laboratory, and this polypeptide was characterized as a bifunctional protein (enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase) in the peroxisomal fatty acid beta-oxidation system, as is the case in the liver of the rat (Baldwin et al., in preparation). This indicates that polypeptide PPA-80 may be used as a parameter to predict the induction of peroxisome proliferation in rainbow trout as well.

Because induction of peroxisomal enzymes does not always parallel the increases of numerical or volume densities of peroxisomes (Lazarow et al., 1982), quantitative morphometric analysis of peroxisomes is suggested as a complementary measurement for peroxisome proliferation. Morphometric analysis of hepatic peroxisomes in rainbow trout supported the findings from the analyses of peroxisomal oxidase, polypeptide PPA-80, and catalase in the present study. While ciprofibrate is reported to increase both volume densities of mitochondria and peroxisomes in some animal species (Reddy et al., 1984), it increased only peroxisomal volume density in rainbow trout. It is of particular interest that peroxisomal volume density of nontreated trout was similar to that of nontreated male rats, as is reported in other studies (Reddy et al., 1984; Berge et al., 1984). In addition, the morphologic analysis showed the absence of a crystalloid core in trout peroxisomes, unlike rodent peroxisomes, which contain the crystalloid core (Reddy and Lalwani, 1983).

The present study was limited in selecting the optimal study period due to the leakage of agents from the injected area and unhealthy conditions of test animals beyond the 3-week exposure. In addition, the selection of the appropriate interval of injection was limited by lack of pharmacokinetic information of the test agents in fish as well as the health status of test animals, which allowed only alternating day injections for the maximum duration of exposure. Despite these limitations, it appears evident that hepatic peroxisome proliferation occurs in liver of rainbow trout receiving 35 mg/kg of ciprofibrate for 3 weeks of alternating day injection. Effects from clofibrate showed induction of peroxisome proliferation based on the elevated peroxisomal oxidase activities. However, the analyses of polypeptide PPA-80, catalase, and morphometry were not done to support this result. While it is speculated that increases of peroxisomal acyl-CoA oxidase in rodents are substantially higher than in trout, baseline data of oxidase activities and peroxisomal volume densities in rainbow trout are in similar ranges to those of rodent species (Reddy et al, 1984; Yang and Leonard, 1988). Thus, it is suggested that the inducibility of peroxisome proliferation in trout may be to a lesser degree than in rodents.

It is unfortunate that the current study cannot be used for a rigorous comparison with other studies, primarily rodent studies, due to the major differences in route of administration, amount of agents used, and duration of exposure. Therefore, it is suggested that further studies using a comparable study design be conducted to test the potential use of trout as an alternative model for making a direct comparison between trout and rodents.

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DEVELOPMENT OF SMALL FISH CARCINOGENESIS BIOASSAYS

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INTRODUCTION

The rationale for this project was based on an expanding body of data suggesting that small fish species can be utilized in rapid, sensitive, economical carcinogenesis bioassays that could enhance the identification of carcinogenic risks that might affect humans or lower organisms. The primary purpose of this research was to examine two small fish species, the Japanese medaka (*Oryzias latipes* Temmick and Schlegel) and the king cobra guppy (*Poecilia reticulata* Peters), for their sensitivity to various classes of carcinogens from the standpoint of neoplasm induction and the ability of the organisms to metabolically activate indirect-acting carcinogens. An ancillary purpose of the research was to examine factors that affect the interpretation of the results of small fish carcinogenesis bioassays. These factors include the occurrence of neoplastic lesions in nontarget organs and the relationship of non-neoplastic lesions to neoplastic processes.

In this presentation, the results of the project with regard to carcinogenic responses of the medaka and guppy to exposure to 1,1,2,2-tetrachloroethane (TeCE), cadmium, and N-2-acetylaminofluorene (2-AAF) are presented, and studies on the hepatic metabolism of 1,2-dibromoethane (EDB) and 2-AAF in the medaka are discussed. Data are also presented on the cumulative spontaneous rate of hepatic neoplastic lesions in the two model species, the relative sensitivity of males versus females to chemically induced hepatic tumorigenesis, and the rates of thymic lymphoma and germ cell neoplasms in the medaka.

EXPERIMENTAL MODELS AND EXPOSURE METHODOLOGY

Studies in this project involve two small fish carcinogenesis models, the Japanese medaka and the king cobra guppy. The two species bring different attributes to the carcinogenesis bioassay. With regard to generating test organisms, the egg-laying medaka has several advantages over the live-bearing guppy. Large numbers, sometimes several thousand, same-day-old medaka embryos can be obtained for testing. Medaka appear to be more genetically uniform than the guppy, and, in concurrent tests, medaka generally appear to be more

sensitive to carcinogens than the guppy. The guppy, on the other hand, appears to have a lower spontaneous tumor rate than the medaka and is more resistant to the toxic effects of some carcinogens. Medaka and guppies develop different types of extrahepatic neoplasms when exposed to the same carcinogen.

With regard to exposure methodology, static, multiple static, and flow-through exposures are employed in the tests. The overall philosophy is to match the most appropriate exposure method with the particular test compound. The type of exposure utilized depends on several factors, including (1) the availability and cost of the test chemical; (2) the quantity and toxicity of the waste that will be generated; (3) the physicochemical characteristics of the test chemical, such as its persistence in water and whether a solvent carrier is needed; and (4) whether the test compound is expected to be carcinogenic following brief exposures or whether prolonged exposures are necessary for carcinogenic activity to be expressed.

Tests usually begin with specimens that are 6 to 12 days old to take advantage of the apparently enhanced carcinogen sensitivity of young fish. Static tests are generally conducted in a carcinogen glove box in 1-liter beakers. Depending on the toxicity and expected carcinogenicity, tests may range from a single 1- to 24-hour exposure to a series of exposures applied on a weekly basis. Chemicals chosen for flow-through tests generally have low water solubility, low expected carcinogenicity, or are tested in that way to mimic some comparable situation such as long-term, low-level exposure of humans to drinking water. Exposures may be as short as 28 days or as long as 6 months. Both static and flow-through tests incorporate the appropriate control groups, including aquarium (out-of-exposure box) controls, flow-through (in-exposure box) controls, and solvent (carrier) controls. Parent compounds are analytically quantified in exposure media to determine actual exposure concentrations over the exposure periods.

HISTOPATHOLOGICAL ENDPOINTS

To date, the principal aim in these studies has been to determine whether the test compounds induce neoplastic lesions in the model organisms. Other factors such as mortality, growth, fecundity, and behavior are measured or observed and related to tumorigenicity. Typically, samples are taken for histopathological analysis at 24 and 36 weeks from the beginning of the exposure, with about 75 to 100 specimens taken at each sampling period. Attempts are made to sample equal numbers of males and females. Indeed, it appears that when the overall results of a study are examined, with specimens taken at several time periods and with all specimens terminated at the final sampling, usually the experimental groups are made up of about 50% males and 50% females. Because these tests are primarily

developmental and are not part of an attempt to confirm or establish test protocols, maximum flexibility in both sampling periods and numbers of specimens sampled at a designated period is maintained. For example, if no tumors are observed in a 24-week sample, the remaining specimens might be apportioned over 36-week and 52-week sampling periods. On the other hand, if substantial numbers of tumors are seen in a 24-week sample, a terminal 36-week sample might be taken. Whole fish specimens are usually processed for histopathology, allowing researchers to examine most major organs and tissues while concentrating on hepatic effects where most positive carcinogens in fish are active.

Generally, carcinogenic responses in fish are seen in hepatic tissues whether or not the initiating or promoting carcinogen is primarily a hepatic carcinogen in other models. For practical analyses of carcinogenesis bioassays with small fish, the most commonly observed carcinogen-induced hepatic lesions are the following: (1) persistent foci of staining alteration (altered foci), (2) hepatocellular adenoma, (3) hepatocellular carcinoma, (4) cholangioma, and (5) cholangiocarcinoma. Persistent altered foci, while they probably do not meet criteria of neoplasms, appear to be specifically carcinogen induced and persist in livers of carinogen-exposed specimens for many months following exposure. They are likely to be preneoplastic and directly or indirectly related to hepatocellular adenoma. Hepatocellular adenoma appears to be a true neoplasm, does not regress, and continues centrifugal and invasive growth for as long as the affected specimen lives. In these studies, it is often difficult to distinguish hepatocellular adenoma from hepatocellular carcinoma. Whereas both have the same neoplastic component, the hepatocyte, the diagnosis of carcinoma is based on the irregularity of the lesion border, the degree of anaplasia of the tumor cells, and the size of the lesion. Cholangiocellular neoplasms, cholangioma and cholangiocarcinoma, which arise from biliary elements in the liver, tend to occur following exposure to toxic levels of carcinogens, and similar to hepatocellular adenoma and carcinoma, are often difficult to distinguish from one another.

The easy identification of extrahepatic neoplasms is a bonus of small fish carcinogenesis studies. In medaka and guppies, extrahepatic carcinogen-induced neoplasms include: retinal neoplasms (medulloepithelioma, pigment epithelial carcinoma); exocrine pancreatic neoplasms (adenoma, carcinoma); neural neoplasms (neurilemmoma, ganglioma); soft tissue neoplasms (fibroma, fibrosarcoma, rhabdomyosarcoma, undifferentiated neoplasms); vascular neoplasms (histiocytoma, hemangioma, hemangiopericytoma); gall bladder epithelial neoplasms (adenoma, carcinoma); renal neoplasms (nephroblastoma, adenocarcinoma); and gill neoplasms (hemangioma, hemangiosarcoma). Other neoplasms occurring at low rates either in controls or in exposed specimens but unrelated to dose or time postexposure include principally thymic

lymphoma and swim-bladder carcinoma. The occurrence of germ cell neoplasms appears to be loosely correlated with exposure to carcinogens or toxic halogenated organics and will be discussed below.

RESPONSES OF MEDAKA AND GUPPIES TO CARCINOGENS REPRESENTING SEVERAL DIFFERENT CLASSES

Because the usefulness of small fish carcinogenesis models hinges on their ability to detect carcinogens representing the widest variety possible, 2-AAF, cadmium, TeCE, and vinylidine chloride are being tested in the medaka and guppy models. To better understand the capabilities of small fish to metabolize precarcinogens to their ultimate carcinogenic forms, biochemical studies are being conducted on the ability of medaka to activate and detoxify 2-AAF, and to activate EDB. Studies on the effects of EDB on alkaline unwinding of DNA in medaka have also been conducted. The hepatic biochemical studies on 2-AAF and EDB have been carried out in collaboration with Dr. Margaret O. James at the C.V. Whitney Marine Biomedical Laboratory, University of Florida, St. Augustine, Florida, and the alkaline unwinding studies with Dr. Lee Shugart, Oak Ridge National Laboratory, Oak Ridge, Tennessee. Studies on the hepatic biochemical effects of EDB in medaka have also been conducted in collaboration with Dr. Braulio Jimenez, Oak Ridge National Laboratory.

N-2-Acetylaminofluorene (2-AAF)

The aromatic amines are a class of chemicals that include the carcinogens benzidine and aniline as well as 2-AAF. Although the carcinogenicity of 2-AAF in rodents is well known and it is widely used as a model carcinogen in initiation-promotion tests, its carcinogenicity, or the carcinogenicity of any other aromatic amine, has not been well documented in fish.

For 2-AAF to be carcinogenic, it must be N-hydroxylated by a cytochrome P-450-dependent, microsomal-bound enzyme. Ring hydroxylation, on the other hand, by another P-450 enzyme appears to be a detoxification step. To investigate the hepatic bioactivation of 2-AAF in medaka, a preliminary 48-hour exposure to 8.6 ppm 2-AAF was run and the activities of a series of mixed-function oxidase (MFO) enzymes were assayed. The activity of the MFO ethoxycoumarin O-deethylase was suppressed by 2-AAF exposure, and a new protein band at approximately 49 kdaltons was observed in the electrophoretic separation of the microsomal fraction of AAF-exposed fish as compared to controls. There was no difference in the bands of the cytosolic fractions of both treatments.

For the definitive study, approximately 100 adult medaka were exposed to 2.1 ppm 2-AAF for 48 hours with 100 untreated medaka serving as controls. Exposed medaka were

transferred to a dilute solution of 2-AAF for 24 hours during which time they were transported to the Whitney Laboratory where they were sacrificed. Incubation of 2-AAF with the microsomal fractions of both treatments resulted in a combined fourfold increase in the amount of combined 2-AAF metabolites in the control treatment compared with that in the 2-AAF pretreated microsomes (Table 1). The major AAF metabolite formed *in vitro* was 7-OH-AAF, followed by 5-OH-AAF, both of which indicate ring hydroxylation. The carcinogenic intermediate, N-OH-AAF, was also produced, demonstrating, at least qualitatively, the activation capability for aromatic amines of medaka hepatic enzyme systems. Results of histopathological examinations are not complete, but early analysis indicates that 2-AAF is not carcinogenic in the medaka but is weakly carcinogenic (incidence about 9%) in livers of the guppy. In summary, 2-AAF depresses hepatic microsomal oxidative enzyme activities whereas it increases glutathione S-transferase activity. Exposure to 2-AAF does not seem to affect the activities of epoxide hydrolase or of glucuronyl transferase. Medaka unexposed to 2-AAF appear to be able to hydroxylate AAF *in vitro* to mainly ring metabolites and to a lesser extent to the N-metabolite, N-hydroxy-AAF, the proximate carcinogen.

1,2-Dibromoethane (Ethylene Dibromide; EDB)

Previous studies in the Gulf Coast Research Laboratory have shown that EDB is highly carcinogenic to medaka, causing a variety of hepatic and extrahepatic neoplasms. Because EDB has an unusual carcinogenic mechanism that involves the Phase II system in activation rather than detoxification, as is usually the case, some studies designed to examine some of those mechanisms were conducted. Adult medaka were exposed to approximately 1 ppm EDB for 14 days in a flow-through system, netted, shipped in exposure water to the Whitney Laboratory, and sacrificed 24 hours later. Livers from the control and exposure groups were pooled into three groups, weighed, homogenized, centrifuged, and treated as separate entities for statistical purposes. Glutathione S-transferase activities (Table 2) and gel electrophoresis of cytosolic proteins were conducted.

Gel electrophoresis of the cytosolic proteins from medaka exposed to 1.3 ppm EDB for 14 days yielded a 26-kdalton protein band that was not seen in specimens exposed to 1.0 ppm EDB for 14 days. In summary, these studies show that EDB exposure results in an increase in glutathione S-transferase activity, possibly increasing the rate of conjugation of glutathione to EDB and increasing the amount of conjugated EDB available for binding to DNA. Other preliminary studies have indicated that FDB exposure in medaka suppresses the activities of hepatic microsomal MFOs that normally metabolize polycyclic aromatic hydrocarbons (Dr.

Table 1. Biotransformation Pathways in Control and AAF-Treated Medaka

	Control	Treated
Protein yield, microsomes	9.64	15.4
mg/g liver cytosol	52	44.4
Oxygenation of AAF		
Total, pmole/min/mg protein	256	68.5
7-OH	210	48.4
5-OH	20	8.8
3-OH	4	1.9
1-OH	2	1.3
N-OH	5	0.7
Glucuronyl transferase, pmole/min/mg protein		
4-Methyl umbelliferone	556	680
3-Hydroxy AAF	181.2	244.6
Epoxide hydrolase (styrene oxide), nmole/min/mg protein	1.51	1.11
GSH-S transferase, nmole/min/mg protein	1,370	1,814
Sulfotransferase, pmole/min/mg protein		
4-Methyl umbelliferone	129.6	40
3-Hydroxy AAF	25.2	30
N-Hydroxy AAF*	184	75

*Results not reliable--assay problems.

Table 2. Glutathione S-Transferase Activities in Japanese Medaka Exposed to 1 ppm EDB for 14 Days (Activities Expressed as nmole/min/mg Protein)

Liver Pool	Control	EDB Exposed
1	5,501	6,353
2	5,814	6,708
3	3,984	7,475
Mean values	5,100	6,845
Standard deviation	±979	±573

Braulio Jimenez, Oak Ridge National Laboratory, personal communication). MFO suppression by EDB could have important implications in assessing the carcinogenicity of mixtures with the medaka model.

Cadmium

Cadmium is a heavy contaminant of air, food, and water supplies. It induces malignant transformation in cultured cells, sarcomas at infection sites in rats, and lung tumors in rodents when administered as an aerosol. Cadmium exposure has been correlated with a high incidence of prostatic and renal cancer in workers occupationally exposed to the metal. Preliminary histopathological analyses of cadmium-exposed medaka have yet to reveal any carcinogenic activity of the metal.

1,1,2,2-Tetrachloroethane (TeCE)

TeCE is a solvent used in cleaning processes and in the manufacture of paints, varnishes, and rust removers. It is used as a soil sterilizer, a weed killer, and an insecticide and in the manufacture of trichloroethylene and other chlorinated hydrocarbons. It has induced hepatocellular carcinoma in mice but not in rats. However, these studies indicate that TeCE is not carcinogenic to medaka and guppy.

FACTORS INFLUENCING THE INTERPRETATION OF SMALL FISH CARCINOGENESIS STUDIES

Whereas in most small fish carcinogenesis studies the primary endpoint has been the development of hepatic neoplastic lesions, pathological factors that confound interpretation and possibly the genesis of those lesions have not been considered or reported in detail. Such factors would include the occurrence of spontaneous (not induced by the test compound) neoplastic lesions, the occurrence of non-target-organ (rare) neoplastic lesions that may or may not be related to chemical exposure, parasitic and microbial infections, pathological changes associated with diet and aging, and nonspecific idiopathic lesions. Here, the occurrence of thymic lymphoma, an apparently spontaneously occurring lesion in medaka, is reviewed.

Thymic Lymphosarcoma

Thirty-nine cases of lymphoma have been diagnosed in a variety of bioassay evaluations involving the Japanese medaka. Twenty-five cases occurred among specimens exposed to test compounds, including 7,12-dimethylbenzanthracene (DMBA), tributyltin oxide, a mixture of

trihalomethanes, TeCE, EDB, and benzo[e]pyrene. Fourteen cases were among fish designated as control specimens for these studies. Lymphoma occurrence in exposed specimens could not be related to the specific compound tested, to the exposure concentration, or to the sex of the individual affected. On two occasions, multiple cases of lymphoma occurred among specimens held in a single aquarium, three cases in one aquarium and two cases in another. The earliest case was diagnosed in a 22-week-old specimen.

If the number of specimens at risk of developing lymphoma is defined as the total number examined after the earliest case was identified, then the overall incidence was 39 out of 9,801 (0.40%), with a rate of 0.44% in chemically exposed specimens and 0.34% in control specimens. Histologically, the lesion was dominated by a small lymphocytic cell with a large basophilic nucleus surrounded by a thin rim of cytoplasm. Mitotic figures were frequently observed. Preliminary electron microscopic studies showed that the nucleus was highly invaginated but revealed no virus particles. Lymphomas appeared to originate in the thymus and infiltrate, in a time-dependent fashion, into the kidney and other retroperitoneal structures, the pharyngeal and cranial regions, and the abdominal viscera. In late stages, the condition became leukemic, and nests of neoplastic cells were seen attached to the muscular trabeculae of the cardiac atrium. Given the scattered occurrence of the thymic lymphomas and the fact that they tend to occur in specimens in contact with one another, a viral etiology is suspected, and studies are planned to confirm this. In any case, thymic lymphoma is apparently one of the most frequently occurring spontaneous neoplasms in the Japanese medaka and should be taken into account when assessing the results of carcinogenesis bioassays.

THE MEDAKA CARCINOGENESIS MODEL

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INTRODUCTION

A general status report on the development of the medaka carcinogenesis model was submitted to the Army by the Environmental Research Laboratory (ERL)-Duluth in August 1988. In that report, the rationale for using small fish in carcinogenesis testing was outlined, the medaka bioassay protocol being used by ERL-Duluth to determine the sensitivity of medaka to various known chemical carcinogens (validation bioassays) was described, the 47 carcinogens and 13 noncarcinogens selected by a chemical selection committee for testing in the medaka bioassay were listed, the chemical selection criteria were outlined, a status report on the progress of chemicals in intermediate stages of the bioassay was provided, and preliminary pathology reports on hexachlorobenzene, aniline, 4-chloroaniline, and di(2-ethylhexyl)phthalate (DEHP) were included. This presentation will include a review of the status of all the bioassays in progress, a description of target organ pathology for several chemicals, and a discussion of the progress that has been made in studying peroxisome proliferation in hepatocytes of medaka chronically exposed to DEHP.

VALIDATION BIOASSAYS

Introduction

A very short review of the rationale for this work and a brief description of the bioassay protocol are necessary to place this work in perspective. The experience of the National Toxicology Program (NTP) demonstrates at least two things. First, *in vitro* tests do not reliably predict *in vivo* results (Tennant et al., 1987), and, second, *in vivo* tests using mammalian models are extremely expensive. Consequently, ERL-Duluth and other laboratories are exploring the use of medaka (*Oryzias latipes*), a small aquarium fish, as a more cost-effective carcinogenesis model. Specific products of this research at ERL-Duluth include:

- Bioassay methods that will predict the relative carcinogenic hazard posed by individual chemicals

- Dose estimates (total body and blood levels) required to produce tumors in medaka
- Direct and indirect evidence that certain carcinogenic mechanisms exist in medaka.

This research, coupled with results from other laboratories, will lead to the development of more cost-effective methods for studying biological dose-response carcinogenesis models. These dose-response models will provide the basis for better risk assessments.

The first and most important task in this research project is to strengthen the premise that medaka are useful organisms for modeling carcinogenesis. To achieve this, the sensitivity and specificity of medaka to known carcinogens and noncarcinogens are being tested with a standardized bioassay. The bioassay protocol established for this task uses an exposure phase followed by a grow-out phase. In the exposure phase, medaka fry (1 to 3 days old) are exposed via water in a flow-through diluter to the test chemical for 28 days. In the grow-out phase, the exposed and control fish are reared in clean water for 5 additional months. At the end of grow-out, when the fish are 6 months old, the fish are sacrificed and prepared for histological analysis.

Animal groups exposed to each of five different concentrations of the test chemical and clean water controls comprise the six exposure groups. Each group is duplicated, with 60 fish in each duplicate. Not all exposure groups are transferred into grow-out. To standardize which groups are transferred, first the maximum tolerated concentration (MTC) for the test chemical is established. The MTC is defined as the highest exposure concentration with not more than 10% mortality compared to the controls. As a minimum, the following groups are moved into grow-out: MTC, 0.5 MTC, and controls. Survivors from concentrations that exceed the MTC, if any, are also moved into grow-out for possible histological study, but may not be included in the statistical analysis.

The pathology analysis is performed first on the fish from the MTC and the controls. If no neoplasia is recorded, then the analysis is complete after the controls are read. If neoplastic lesions are observed in fish from the MTC, then fish from the 0.5 MTC are also analyzed. Subsamples of both replicates of all exposure groups are taken at the end of the 28-day exposure and are prepared histologically to study target organ pathology. All moribund fish in grow-out are also prepared for histological analysis.

Histological samples are either fixed in 10% buffered formaldehyde and embedded in glycol methacrylate or fixed in Bouin's solution and embedded in paraffin. The tissue blocks are step-sectioned parasagittally and stained in hematoxylin and eosin. Parasagittal step-sectioning allows pathological analysis to be done on 31 selected tissues, although all of these tissues may not be present in the slides produced for each specimen. For statistical purposes,

at least 60 fish are analyzed for neoplastic lesions from the MTC and control groups. This sample size allows detection of, with 95% confidence, at least one tumor if the chemical causes a 5% tumor incidence in any group.

After many chemicals have been analyzed using the protocol reviewed above, concordance of the medaka carcinogenesis model to other *in vivo* carcinogenesis models can be studied. Concordance, at least superficially, can be interpreted to infer model similarity. This is a powerful inference as it suggests that all the stages of a complex multistep process are present in the medaka. Discordance is not as easily interpreted. If, for instance, the response of the medaka bioassay is falsely negative, then one of two conclusions can be drawn: either the medaka itself is an inadequate model or the specific bioassay protocol is inadequate.

To address the issue of false negative results with known carcinogens, an iterative approach to the empirical validation procedure will be used. Certain positive animal carcinogens that are negative in the standard protocol outlined above will be rerun using a revised protocol. The major protocol revision will be to prolong the exposure time up to 6 months. Included in this protocol revision will be periodic sampling throughout the exposure for histopathology. Also, for hydrophobic chemicals with low water solubility (i.e., chemicals with a high log P), total body residue analyses will be performed through time to determine when steady-state concentrations of the carcinogen are achieved. In those cases where waterborne high log P chemicals do not achieve a high enough concentration in the fish to be carcinogenic, alternative exposure methods, such as microencapsulation of compounds in food, will be considered.

Progress

As a review of the chemicals selected by the chemical selection committee, a list of the 47 carcinogens (Table 1) and a list of the 13 noncarcinogens (Table 2) to be tested in the medaka carcinogenesis bioassay are included. Many of the chemicals in Table 2 are noncarcinogenic analogs of the carcinogens in Table 1. Table 3 shows the overall status of chemicals on which work has been done. Table 4 shows the end of exposure toxicity results, including the measured aqueous concentration and the percent survival for each exposure and control tank.

Table 1. Carcinogenic Chemicals Chosen for Validation of the Medaka Carcinogenicity Assay*

	CAS Number	Chemical
1	50000	Formaldehyde
2	50180	Cyclophosphamide
3	50293	p,p'-DDT
4	50555	Reserpine
5	51525	6-Propyl-2-thiouracil
6	51752	Nitrogen mustard
7	51796	Urethane
8	55801	3'-Methyl-4-dimethylaminoazobenzene
9	56531	Diethylstilbestrol
10	57067	Allyl isothiocyanate
11	57147	1,1-Dimethylhydrazine
12	57578	beta-Propiolactone
13	67210	dl-Ethionine
14	71432	Benzene
15	78591	Isophorone
16	91598	2-Naphthylamine
17	95807	2,4-Diaminotoluene
18	96457	N,N'-Ethylenethiourea
19	100754	N-Nitrosopiperidine
20	106934	Ethylene dibromide; (1,2-Dibromoethane)
21	107073	2-Chloroethanol
22	117817	Di(2-ethylhexyl)phthalate (DEHP)
23	118741	Hexachlorobenzene
24	122667	Hydrazobenzene
25	123911	1,4-Dioxane
26	126727	Tris(2,3-dibromopropyl)phosphate
27	134292	o-Anisidine hydrochloride
28	140114	Benzyl acetate
29	140885	Ethyl acrylate
30	150685	Monuron
31	154938	Bis-N-N'-(chloroethyl)-nitrosourea
32	512561	Trimethyl phosphate
33	531851	Benzidine hydrochloride
34	540738	1,2-Dimethylhydrazine
35	542756	1,3-Dichloropropene
36	602879	5-Nitroacenaphthene
37	759739	N-Nitroso-N-ethylurea
38	1746016	2,3,7,8-TCDD
39	3771195	Nafenopin
40	5131602	4-Chloro-m-phenylenediamine
41	7227910	1-Phenyl-3,3-dimethyltriazene
42	7488564	Selenium sulfide
43	7789062	Strontium chromate
44	10034932	Hydrazine sulfate
45	12035722	Nickel subsulfide
46	24554265	N-[4-(5-Nitro-2-furyl)-2-thiazolyl] formamide
47	26471625	2,4 & 2,6-Toluene diisocyanate mix

*These chemicals were chosen by a committee of scientists from EPA and the National Toxicology Program, September 1987.

Table 2. Noncarcinogenic Chemicals Chosen for Validation of the Medaka Carcinogenicity Assay*

	CAS Number	Chemical
1	54886	2-Methyl-4-dimethylaminoazobenzene
2	72435	Methoxychlor
3	77656	Carbomal
4	91203	Naphthalene
5	95501	1,2-Dichlorobenzene
6	108952	Phenol
7	109693	Chlorobutane
8	116063	Aldicarb
9	134327	1-Naphthylamine
10	148243	8-Hydroxyquinoline
11	333415	Diazinon
12	624180	p-Phenylenediamine dihydrochloride
13	2481949	N,N-Diethylaminoazobenzene

*These chemicals were chosen by a committee of scientists from EPA and the National Toxicology Program, September 1987.

TARGET ORGAN PATHOLOGY

Introduction

As part of the current protocol used in the medaka carcinogenicity assay, fish are sampled from each exposure concentration for histological analysis at the end of the 28-day exposure. These fish are assessed for target organ pathology. Determination of target organ effects will be used to ascertain if the toxicity of a chemical is organ specific, if organ specificity correlates with mammalian data, and if tissues affected at the end of exposure are the same tissues that become neoplastic at the end of the grow-out. Included in this report are the results of the 28-day target organ pathology for the following chemicals: hexachlorobenzene, aniline, 4-chloroaniline, formaldehyde, 1,1-dimethylhydrazine, ethyl acrylate, 1,4-dioxane, and 2,4-diaminotoluene.

Materials and Methods

Fish were exposed using the standard 28-day protocol for seven of the eight chemicals in this study (see exposure description in the section on validation bioassays in this report). The only chemical for which the exposure deviated from the standard protocol was hexachlorobenzene. Hexachlorobenzene was run at aqueous saturation (5.2 μg/liter) for 28 days with only one exposure tank and one control tank. At the end of all exposures, six fish

Table 3. Status of Chemicals Chosen for Validation of the Medaka Carcinogenicity Assay

CAS Num.	Chemical Name	Exposure Planned	In Exposure	In Grow-Out	In Histology	In Analysis
62553	Aniline*					•••
106478	4-Chloroaniline*					•••
118741	Hexachlorobenzene* (28 day)					•••
47147	1,1-Dimethylhydrazine					•••
94757	2,4-D					•••
140114	Benzyl acetate				•••	
117817	DEHP (9 month)				•••	
134292	Anisidine hydrochloride				•••	
106934	1,2-Dibromoethane				•••	
123911	1,4-Dioxane				•••	
50000	Formaldehyde				•••	
107073	2-Chloroethanol				•••	
108952	Phenol				•••	
117817	DEHP (28 day)				•••	
140885	Ethyl acrylate				•••	
51796	Urethane				•••	
95807	2,4-Diaminotoluene				•••	
117817	DEHP (6 month)				•••	
542756	1,3-Dichloropropene				•••	
71432	Benzene				•••	
57067	Allyl isothiocyanate				•••	
116063	Aldicarb				•••	
78591	Isophorone				•••	
512561	Trimethylphosphate			•••		
96457	N,N'-Ethylenethiourea (Imidazolidinethione)			•••		
100754	N-Nitrosopiperidine			•••		

*Exposure began before chemical selection meetings.

Table 3. Status of Chemicals Chosen for Validation of the Medaka Carcinogenicity Assay (continued)

CAS Num.	Chemical Name	Exposure Planned	In Exposure	In Grow-Out	In Histology	In Analysis
118741	Hexachlorobenzene (56 day)			***		
118741	Hexachlorobenzene (6 month)		***			
91598	2-Naphthylamine	***				
531851	Benzidine hydrochloride	***				
67210	dl-Ethionine	***				
50555	Reserpine	***				
56531	Diethylstilbestrol	***				
50293	p,p'-DDT	***				
50180	Cyclophosphamide	***				

Table 4. Summary Data Table for 23 Completed Carcinogenicity Exposures*

CAS #	Chemical Name		Tank	1		2		3		4		5		6	
			Replicate	A	B	A	B	A	B	A	B	A	B	A	B
50000	Formaldehyde	(conc.)		0.02	0.02	2.57	2.26	5.86	5.19	10.6	11.0	21.9	23.8	48.6	47.7
		(% survival)		(95)	(100)	(95)	(98)	(100)	(100)	(93)	(93)	(95)	(95)	(95)	(87)
51796	Urethane			<4.00	<4.00	412	428	559	545	933	906	1870	1910	3860	4040
				(100)	(97)	(97)	(97)	(97)	(97)	(100)	(95)	(85)	(73)	(0)	(0)
57067	Allyl isothiocyanate (ug/l)			<0.50	<0.50	2.62	1.93	5.01	4.96	8.03	8.03	18.3	19.6	42.1	40.4
				(100)	(100)	(97)	(100)	(97)	(98)	(95)	(88)	(30)	(27)	(0)	(0)
57147	1,1-Dimethylhydrazine			<0.10	<0.10	1.51	1.44	2.56	2.32	4.07	4.27	8.31	8.17	16.9	17.3
				(90)	(98)	(100)	(98)	(95)	(95)	(100)	(98)	(45)	(42)	(0)	(0)
62533	Aniline			<2.00	<2.00	4.70	4.50	8.68	8.74	18.0	17.9	36.7	37.1	73.3	73.1
				(98)	(97)	(93)	(90)	(80)	(82)	(60)	(73)	(53)	(45)	(42)	(15)
71432	Benzene			<0.25	<0.25	1.40	0.86	2.12	1.51	3.30	2.23	6.04	4.55	15.0	12.7
				(97)	(97)	(98)	(95)	(90)	(97)	(100)	(98)	(90)	(100)	(65)	(68)
78591	Isophorone			<5.00	<5.00	20.8	18.9	35.6	34.3	61.0	58.1	116	114	228	227
				(98)	(95)	(95)	(98)	(98)	(98)	(100)	(95)	(58)	(65)	(0)	(0)
90040	o-Anisidine Hcl			<0.10	<0.10	6.30	6.53	12.0	12.5	25.2	26.9	54.9	58.0	115	124
				(92)	(97)	(100)	(93)	(97)	(98)	(98)	(95)	(97)	(98)	(87)	(92)
94757	2,4-D			<0.50	<0.50	2.47	2.34	5.76	5.75	13.4	13.7	29.8	30.2	60.6	59.6
				(100)	(100)	(100)	(98)	(95)	(98)	(98)	(100)	(95)	(100)	(90)	(77)
95807	2,4-Diaminotoluene			<0.70	<0.70	39.4	40.5	88.5	88.9	130	131	285	262	563	542
				(97)	(98)	(97)	(92)	(87)	(85)	(40)	(46)	(0)	(0)	(0)	(0)
106478	4-Chloroaniline			<0.50	<0.50	2.30	2.19	4.17	4.12	8.21	8.25	15.7	16.1	30.7	31.5
				(97)	(98)	(98)	(93)	(98)	(100)	(95)	(93)	(93)	(83)	(32)	(37)

*Concentrations are mg/liter unless otherwise noted.

Table 4. Summary Data Table for 23 Completed Carcinogenicity Exposures* (continued)

CAS #	Chemical Name	Tank	1		2		3		4		5		6	
		Replicate	A	B	A	B	A	B	A	B	A	B	A	B
106934	1,2-Dibromoethane	(conc.)	<1.00	<1.00	1.53	1.55	2.59	2.60	5.88	6.09	9.96	9.79	21.2	20.5
		(% survival)	(98)	(100)	(100)	(100)	(100)	(95)	(100)	(100)	(100)	(100)	(32)	(35)
107073	2-Chloroethanol		<0.30	<0.30	1.82	1.96	3.73	3.83	8.27	7.73	15.3	15.4	31.4	31.3
			(98)	(95)	(97)	(98)	(100)	(97)	(98)	(98)	(90)	(90)	(55)	(65)
108952	Phenol		<0.05	<0.05	0.97	1.44	2.36	3.02	5.58	6.49	12.3	12.4	31.0	29.9
			(97)	(100)	(100)	(98)	(100)	(98)	(93)	(98)	(98)	(98)	(0)	(0)
116063	Aldicarb		<6.00	<6.00	28.2	28.3	51.2	50.4	90.8	92.0	166	177	334	345
	(ug/l)		(98)	(97)	(92)	(85)	(88)	(93)	(97)	(98)	(67)	(75)	(7)	(10)
117817	DEHP (28-day)		<25	<25	910	896	--	--	--	--	--	--	--	--
	(ug/l)		(99)	(98)	(97)	(99)								
117817	DEHP (6-month)		81.3	78.4	503	526	--	--	--	--	--	--	--	--
	(ug/l)		(99)	(95)	(89)	(93)								
118741	Hexachlorobenzene		0.01	--	5.20	--	--	--	--	--	--	--	--	--
	(ug/l)		(93)		(91)									
123911	1,4-Dioxane		<50.0	<50.0	547	582	978	1010	1830	1910	3550	3520	6900	6960
			(93)	(97)	(97)	(98)	(100)	(100)	(95)	(93)	(88)	(95)	(83)	(85)
140114	Benzyl acectate		0.00	0.00	0.28	0.27	0.46	0.50	0.95	0.91	1.97	1.93	4.72	5.13
			(98)	(98)	(98)	(93)	(98)	(100)	(100)	(97)	(22)	(17)	(3)	(10)
140885	Ethyl acrylate		<0.07	<0.07	0.18	0.19	0.32	0.29	0.52	0.50	1.04	1.02	2.10	2.01
			(88)	(97)	(95)	(100)	(98)	(100)	(95)	(100)	(33)	(28)	(?)	(2)
512561	Trimethylphosphate		<30.0	<30.0	403	437	593.	583	809	891	1420	1420	2640	2740
			100	100	100	100	100	100	100	100	97	92	78	53
542756	1,3-Dichloropropene		<0.01	<0.01	0.13	0.14	0.25	0.27	0.53	0.53	1.17	1.33	2.68	2.80
			(98)	(97)	(100)	(92)	(97)	(97)	(97)	(98)	(75)	(75)	(0)	(0)

*Concentrations are mg/liter unless otherwise noted. --, Double hyphens indicate no exposure group.

from each exposure concentration and control were randomly sampled, processed histologically, and analyzed for histopathology.

Tissues were examined by light microscopy for histopathology. If a pathological effect was observed, a severity rating was assigned according to the following scale:

0 no effect
1 minimal effect
2 minimal-mild effect
3 mild effect
4 mild-moderate effect
5 moderate effect
6 moderate-severe effect
7 severe effect.

The scoring was performed by one individual for all specimens from the same chemical exposure. The scores are only comparable within each exposure group and represent relative severity. The severity was averaged for each tissue affected in each group. In addition, frequency (%) was calculated for each lesion.

Results

Hexachlorobenzene did not produce significant growth or survival effects at 5.2 μg/liter after 28 days of exposure (Table 4). Serious target organ pathology was not noted, except for a qualitative decrease in hepatic glycogen (based on PAS positive staining) and an increase in hepatic lipid content (based on Azure B staining). The lack of any signs of toxicity indicates that medaka did not accumulate a toxic residue and that the exposure was inadequate to elicit identifiable target organ effects.

Aniline produced pathologies that were noted in the following four tissues: gas bladder, liver, kidney, and erythrocytes (Table 5a). The gas bladder pathology was diagnosed as an epithelioma and occurred in the four highest concentrations. The epithelioma was severe in all cases. The frequency ranged from 17% in the 8.85 mg/liter exposure to 83% in the 37.50 and 74.30 mg/liter exposures. The frequency of this lesion was concentration dependent, while the severity was concentration independent (Table 5b). All other lesions were non-neoplastic in nature. The kidney showed signs of glomerular degeneration, indicated by hyaline droplet accumulation in the epithelium of the renal tubules. Hyaline droplet formation is caused by the uptake of protein lost across the glomerulus. Additionally, hemosiderin accumulation in the hemopoietic tissue of the kidney and spleen indicated that aniline caused erythrocyte toxicity. The liver had focal areas of necrosis and dilation of the sinusoidal spaces. Qualitatively, the frequency of these lesions was concentration independent, while the severity

Table 5a. Aniline target tissue effects. *

Tissue	Effect
Gas Bladder	Epithelioma
Kidney	Glomerular degeneration Hyaline droplet formation
Liver	Dilated sinusoids Necrosis
Erythrocytes	Hemosiderin accumulation

*Please note that in Tables 5 through 9, all of the (a) tables list the observed pathologies, while the (b) tables include the measured concentration (mg/l), survival (%) at the end of 28 days of exposure, and a column for each target organ. Under each target organ there are two numbers for each exposure concentration. The upper number indicates the percentage of the group affected with the target organ effect, while the lower number indicates the mean severity of the lesion based on a relative scale of 0 (no effect) to 7 (severe effect).

Table 5b. Aniline target tissue dose response.

mg/l	% Survival	$\bar{X}$ Weight (mg)	Gas bladder	Kidney	Liver	Erythrocytes
<1.00	98	31	0	0	0	0
			0	0	0	0
4.67	92	*18	0	83%	100%	100%
			0	2	4	4
8.85	*81	*14	17%	100%	83%	100%
			7	3	5	5
18.20	*67	*8	66%	66%	83%	100%
			7	5	5	5
37.50	*49	*7	83%	100%	83%	100%
			7	5	5	6
74.30	*28	*4	83%	100%	100%	100%
			7	5	6	5

* Significantly different than controls ($p \leq 0.05$)

was concentration dependent. The kidney, erythrocyte, and liver lesions were all noted at the lowest concentration tested, 4.67 mg/liter, which also caused significant growth reduction (Table 5b).

4-Chloroaniline produced pathologies that were noted in the following four tissues: kidney, erythrocytes, liver, and gill (Table 6a). 4-Chloroaniline acted similarly to aniline in producing the same pathologies of the kidney and erythrocytes. Only erythrocyte toxicity was noted in all fish from all five exposure concentrations. It was the only pathology found at the lowest concentration of 2.34 mg/liter. Its frequency, therefore, was concentration independent, while the severity of this pathology was concentration dependent (Table 6b). The liver pathologies caused by 4-chloroaniline were markedly different from those caused by aniline. These included: atypical liver morphology (in some cases this was typified by a complete loss of normal hepatic architecture), pleiomorphic cells and nuclei, and areas of vacuolation and necrosis. The frequency and severity of these effects were both concentration dependent. Lesions of the gill lamellae included: lamellar fusion, epithelial hypertrophy, and atypia (Tables 6a and 6b). In interesting contrast to aniline, no lesions at all were noted in the gas bladder.

Formaldehyde did not cause any target organ pathologies in the highest exposure concentration, 48 mg/liter. There were also no significant growth or survival effects at this concentration. Therefore, the MTC was not established in this exposure due to two unusual toxic properties of formaldehyde to medaka. First, the dose-response curve, with respect to mortality, is very steep. This results in one concentration causing complete mortality, while the adjacent lower concentration causes no toxic effects at all. Second, formaldehyde is more toxic to older fish than the young fish with which the exposure begins. Consequently, the MTC is time dependent and changes throughout the duration of the exposure, making it difficult to interpret or predict. With longer exposure times at 48 mg/liter or with a higher concentration for the 28-day exposure, target organ pathology may have developed.

1,1-Dimethylhydrazine produced pathologies specifically in the eye. The eye pathologies included retinal malformation, proliferation of retinal stem cells, and reduction in the surrounding pigmented epithelium (Table 7a). The absence of these effects in the lower two concentrations and the increasing frequency and severity of these pathologies in the two highest concentrations suggest that both frequency and severity were concentration dependent (Table 7b). More recent analyses of the retinal lesion suggest that it may be neoplastic.

Ethyl acrylate produced no pathology in the highest concentration with survivors, 1.03 mg/liter. This was unexpected because growth and survival were both reduced by 70%.

Table 6a. 4–Chloroaniline target tissue effects.

Tissue	Effect
Gill	Lamellar fusion Atypical morphology Epithelial hypertrophy
Kidney	Hyaline droplet formation Tubular degeneration Glomerular degeneration
Liver	Atypical morphology Pleiomorphic cells and nuclei Necrosis Vacuolation
Erythrocytes	Hemosiderin accumulation

Table 6b. 4–Chloroaniline target tissue dose response.

mg/l	% Survival	$\bar{X}$ Weight (mg)	Gill	Kidney	Liver	Erythrocytes
<0.5	81	36	0	0	0	0
			0	0	0	0
2.34	79	*24	0	0	0	100%
			0	0	0	1
4.31	85	*20	0	0	33%	100%
			0	0	1	1
8.57	76	*16	66%	33%	100%	100%
			4	1	3	3
16.50	*65	*12	100%	100%	100%	100%
			4	2	4	3
32.40	*36	*5	100%	100%	100%	100%
			6	2	7	4

* Significantly different than controls ($p \leq 0.05$)

Table 7a. 1,1-Dimethylhydrazine target tissue effects.

Tissue	Effect
Eye	Retinal malformation (developmental) Proliferation of retinal stem cell Reduced pigment layer

Table 7b. 1,1-Dimethylhydrazine target tissue dose response.

mg/l	% Survival	$\bar{X}$ Weight (mg)	Eye
<0.10	94	57	0 0
1.48	99	51	0 0
2.44	95	64	0 0
4.17	99	59	40% 4
8.24	*44	*24	100% 7
17.1	*0	--	-- --

* Significantly different than controls ($p \leq 0.05$)

1,4-Dioxane produced pathological effects only in the kidney. These pathologies included: glomerular degeneration, tubular degeneration, and hyaline droplet accumulation in the epithelium of the renal tubules (Table 8a). The absence of these lesions in the lower two concentrations and the increase in both frequency and severity in the highest three concentrations suggest that these effects were concentration dependent (Table 8b).

2,4-Diaminotoluene produced pathologies in the following tissues: erythrocytes, kidney, and liver (Table 9a). The erythrocyte pathology was typified by nuclear anomalies, including: pyknosis, fragmentation (formation of micronuclei), bi-lobed profiles, and general nuclear pleomorphism. All fish in the three exposure concentrations with survivors were affected. The severity of the effect increased with concentration. The kidney pathologies included tubular degeneration and hyaline droplet accumulation in the epithelium of the renal tubules. The liver pathologies included hepatocyte atypia, necrosis, and cytoplasmic degeneration. Both liver and kidney pathologies were present in all exposure concentrations with survivors (Tables 9a and 9b).

Discussion

The specificity of target organ toxicity is determined by many factors, including local physiological conditions, pharmacokinetic distribution, metabolic and detoxification pathways, and route of exposure (Cohen, 1988; Doull, 1980). The identification of target organ effects by exposure to a chemical implies that one or more of the above factors plus attributes of the chemical itself cause a unique combination of events that leads to site-specific toxicity. Analyzing the sites and the types of pathologies elicited by xenobiotics can provide data that will help elucidate mechanisms of cellular toxicity. Data from this study suggest, for instance, that direct water exposure usually does not affect the gill adversely, even though it is the principal route of uptake. In fact, two of the eight chemicals, 1,1-dimethylhydrazine and 1,4-dioxane, affected single tissues (the eye and the kidney, respectively) that seemingly have no direct relation to the route of exposure. Only one chemical, 4-chloroaniline, directly affected the gill.

Three chemicals--hexachlorobenzene, formaldehyde, and ethyl acrylate--did not produce pathological lesions. Because there were no effects on survival or growth in the hexachlorobenzene and formaldehyde exposures, the exposures were insufficient to reach any conclusion. For hexachlorobenzene, this was due to the low water solubility of hexachlorobenzene (5.2 μg/liter), which in turn did not accumulate in the fish sufficiently to cause a toxic residue in the 28-day exposure period. A longer exposure might allow the accumulation of a toxic residue and make it possible to identify target organ pathology.

Table 8a. 1,4–Dioxane target tissue effects.

Tissue	Effect
Kidney	Hyaline droplets Glomerular degeneration Tubular degeneration

Table 8b. 1,4–Dioxane target tissue dose response.

mg/l	% Survival	$\bar{X}$ Weight (mg)	Kidney
<50.0	95	55	0 0
565	98	47	0 0
994	100	54	0 0
1870	94	53	33% 1
3536	92	*35	83% 3
6933	*84	*10	83% 4

* Significantly different than controls ($p \leq 0.05$)

Table 9a. 2,4–Diaminotoluene target tissue effects.

Tissue	Effect
Kidney	Tubular degeneration Hyaline droplet formation
Liver	Atypical morpholgy Necrosis Cytoplasmic degeneration
Erythrocytes	Nuclei pleiomorphic, pyknotic, fragmented, bi–lobed

Table 9b. 2,4–Diaminotoluene target tissue dose response.

mg/l	% Survival	$\bar{X}$ Weight (mg)	Kidney	Liver	Erythrocytes
<0.7	98	43	0 0	0 0	0 0
46	94	*20	50% 4	100% 6	100% 3
78	*86	*14	50% 3	83% 6	100% 4
148	*44	*6	83% 4	83% 7	100% 7
300	*0	--	-- --	-- --	-- --

* Significantly different than controls ($p \leq 0.05$)

Formaldehyde, on the other hand, has an unusually steep mortality curve, which also has an unusual age dependence. The concentration that elicits a toxic response without causing complete mortality for a given exposure length and fish age needs to be established. Ethyl acrylate, however, was toxic; at a concentration where growth and survival were reduced by 70%, no pathologies were noted.

The data in this study demonstrate that although some of the chemicals affected the same organs, the specificity of the response was qualitatively quite different. Aniline, 4-chloroaniline, and 2,4-diaminotoluene all affected multiple tissues. Aniline, however, was the only chemical to affect the gas bladder, causing epitheliomas in all but the lowest exposure concentration. Aniline and 4-chloroaniline caused similar toxicity to the erythrocytes, as was demonstrated by hemosiderin accumulation in the hemopoietic tissue. While 2,4-diaminotoluene also affected the erythrocytes, its effect was strikingly different, causing a wide array of nuclear anomalies. All three chemicals affected the liver, but 4-chloroaniline was the only chemical that caused fundamental changes in liver architecture.

The data in this study also demonstrate that the histopathology endpoint is sensitive. Two of the five chemicals that caused pathology--1,1-dimethylhydrazine and 1,4-dioxane--did so at lower exposure concentrations than those concentrations in which growth was significantly reduced. The other three chemicals--aniline, 4-chloroaniline, and 2,4-diaminotoluene--caused pathology at the lowest exposure concentration. These concentrations also caused significant growth reductions. To determine which endpoint is more sensitive would require an additional assay at lower exposure concentrations.

In summary, this study demonstrates that target organ toxicity can be evaluated successfully in medaka using histopathology. This endpoint requires relatively short exposure times (28 days) and relatively small sample sizes ($n = 6$ for each treatment). Furthermore, for the six chemicals in this study that reduced growth, histopathology was more sensitive in two cases and as sensitive in three cases.

The target organ responses observed in this study should be compared to the mammalian responses to determine if the same target organs are affected. If these relationships are concordant, then the efficacy of an aqueous exposure to fish as the basis for a comparative toxicity or carcinogenicity model will be strengthened.

Finally, these results should be reviewed along with the carcinogenicity endpoint, which is typically ascertained after 5 additional months of grow-out. When these data are available, the 28-day target organ pathology may provide insight about tissue-specific carcinogenicity at 6 months.

PEROXISOME PROLIFERATION IN MEDAKA CHRONICALLY EXPOSED TO DI(2-ETHYLHEXYL)PHTHALATE (DEHP)

Introduction

Carcinogens are sometimes classified into two broad categories: genotoxic and nongenotoxic. Among those classified by *in vitro* tests as nongenotoxic are phthalate ester plasticizers such as DEHP and hypolipidemic drugs such as clofibrate (Reddy et al., 1983). The mechanism by which these chemicals are carcinogenic remains unknown, but in mammals it is associated with the proliferation of cellular organelles called peroxisomes (Reddy et al., 1980).

Peroxisomes are single-membrane limited cytoplasmic organelles that are thought to be involved in gluconeogenesis, oxidation of fatty acids, and the detoxification of hydrogen peroxide. Chemicals that markedly increase the number of peroxisomes are termed "peroxisome proliferators" and, as a class, may be carcinogenic (Reddy et al., 1980; Svoboda and Azarnoff, 1979).

As an adjunct study to a validation bioassay described previously, it was decided to explore the sensitivity of medaka to a model peroxisome-proliferating compound, DEHP. The objective of this research was to determine if DEHP intoxication of medaka causes peroxisome proliferation in the liver.

Materials and Methods

To easily discriminate peroxisomes from other cellular microbodies such as lysosomes, a histochemical procedure was used to specifically stain peroxisomes with 3,3'-diaminobenzidine tetrahydrochloride (DAB). DAB and osmium tetroxide form a light and electron-dense complex specific to catalase reaction products. Catalase is the marker enzyme specific to peroxisomes.

Fifty medaka (30 days old) were placed into each of two aquaria in a flow-through exposure system. Lake Superior water at 25°C was delivered to the control aquarium. The exposure aquarium was identical to the control aquarium with the exception that incoming water contained DEHP at saturation (400 ppb). The fish were maintained on a 16/8 light cycle and fed twice daily with live brine shrimp.

After 45 days of exposure, five fish were removed from each aquarium and anesthetized in MS-222. The livers were fixed in 0.1 M sodium cacodylate buffered 2.5% glutaraldehyde. Following fixation, the livers were stained en bloc with DAB for 45 minutes at 37°C (Novikoff and Goldfischer, 1969). After staining with DAB, the tissues were postfixed in 1% osmium

tetroxide at 4°C for 90 minutes, dehydrated in graded ethanol solutions, and embedded in Araldite 502. Blocks were sectioned at 1.5 μm on a Sorvall MT2-B ultramicrotome.

Results and Discussion

Examination by light microscopy of liver sections from exposed fish revealed numerous dark-staining microbodies. Further analysis by electron microscopy determined that these microbodies were roughly spherical in shape and approximately 0.1-1.5 μm in diameter (Figure 1), corresponding to the morphological characteristics reported for peroxisomes (Beard and Novikoff, 1969). The presence of a crystalloid core (Figure 2) in many of the microbodies identified them unequivocally as peroxisomes (Novikoff and Goldfischer, 1969).

Control and exposed tissues from all fish sampled were examined by light and electron microscopy. Qualitative evaluations showed an overall increase in the numbers of peroxisomes in hepatocytes from exposed fish compared to hepatocytes from controls (Figure 3 and Table 10). These results have not been quantified.

Plans for future research include completion of a test in which the apparent proliferation of peroxisomes induced by exposure to DEHP will be quantified using image analysis techniques on a larger number of samples. Also, because hepatomegaly has been reported in rats and mice following exposure to a peroxisome proliferator (Lazarow et al., 1982), the body weights and liver weights from all fish sampled will be measured to determine if DEHP increases the hepatosomatic index. Finally, the remaining fish will be held in grow-out after approximately 6 months of exposure and examined for evidence of increased neoplasia. This information will be useful in assessing the ability of medaka to respond to peroxisome-proliferating chemicals.

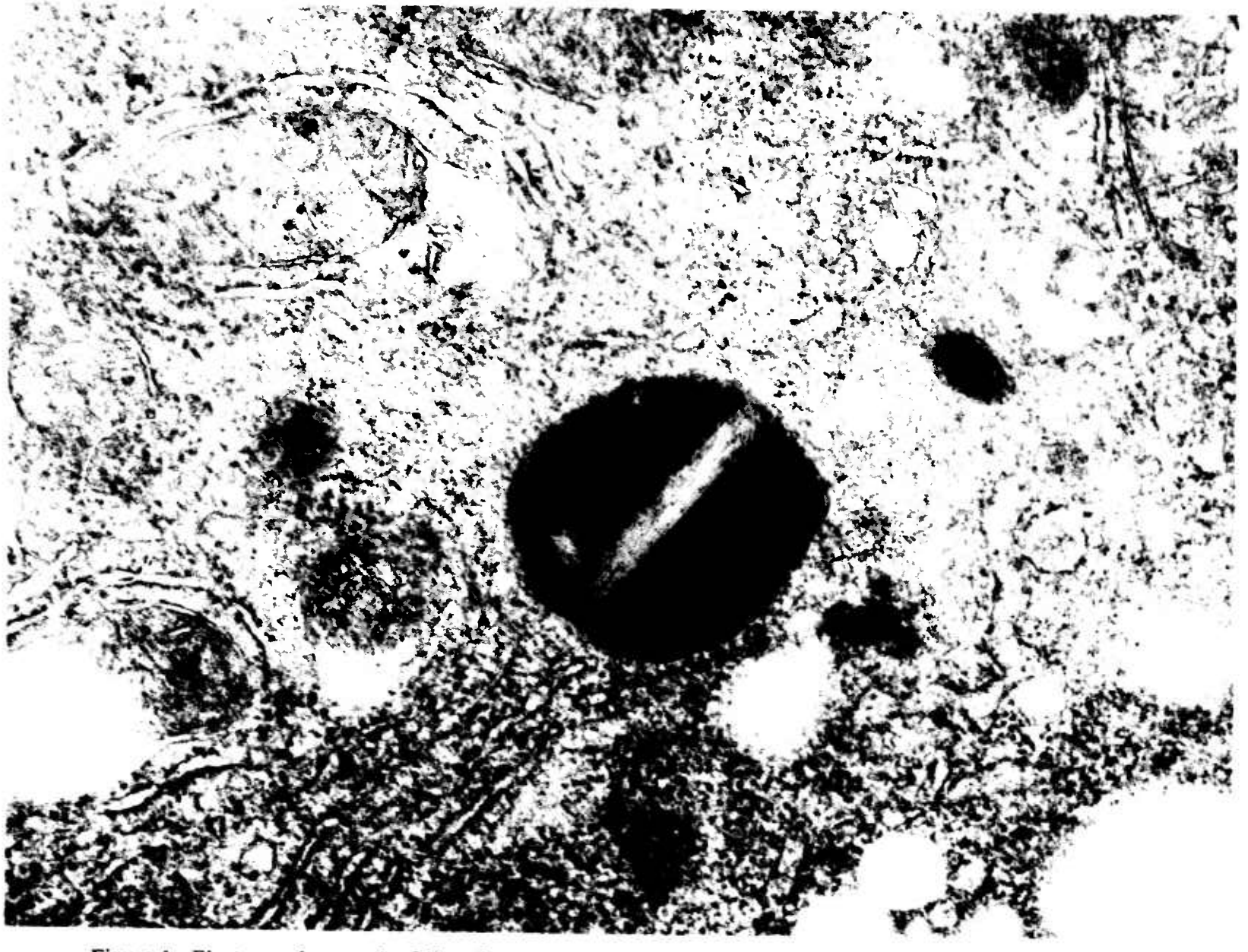

Figure 1. Electron micrograph of liver from medaka exposed to DEHP. The three darkly stained organelles are peroxisomes. (DAB stained; 80,000x.)

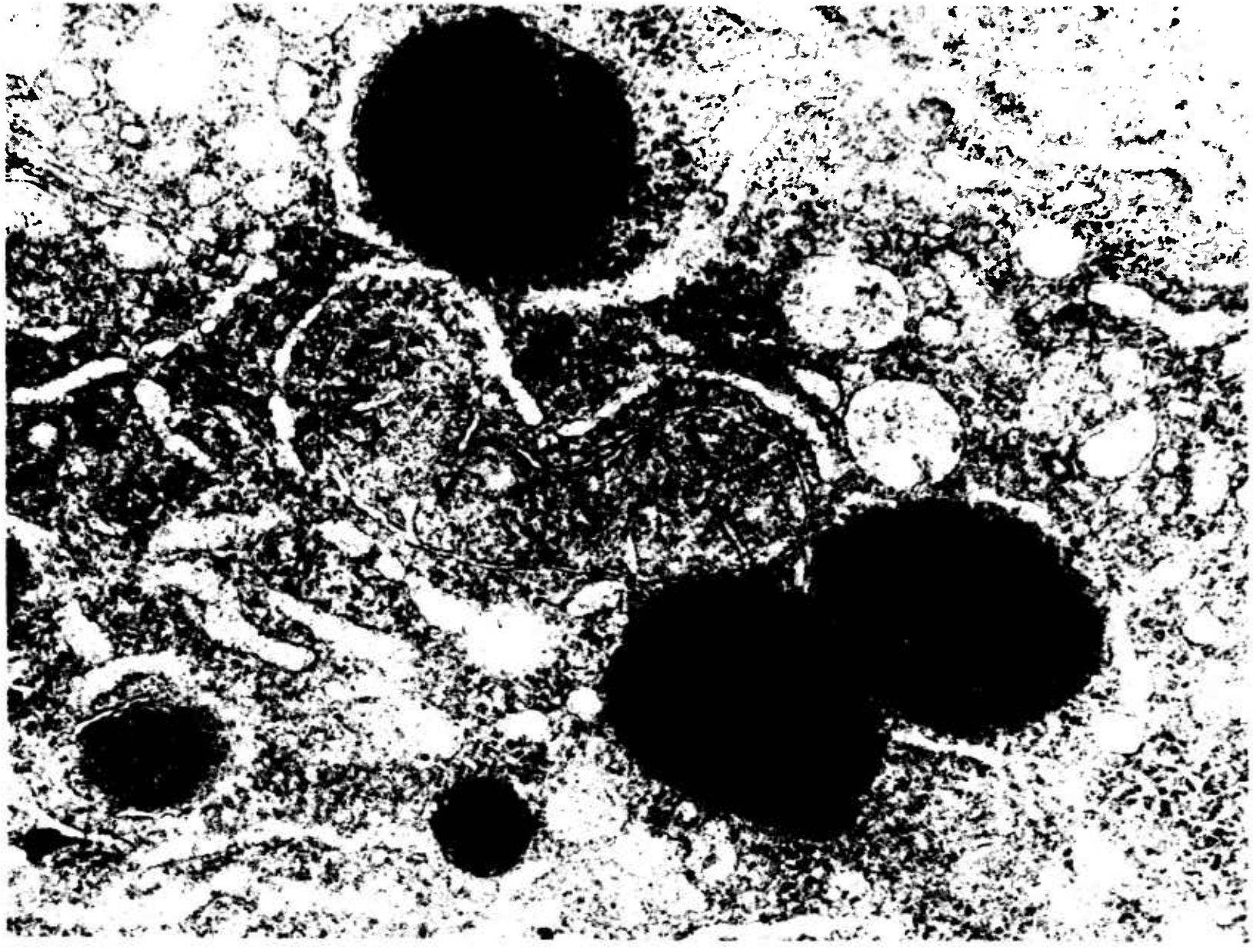

Figure 2. Electron micrograph of liver from medaka exposed to DEHP. The darkly stained organelle in the center of the micrograph is a peroxisome. Note the crystalloid core of the peroxisome. This core is thought to be the enzyme uricase and positively identifies the peroxisome. (DAB stained; 133,000x.)

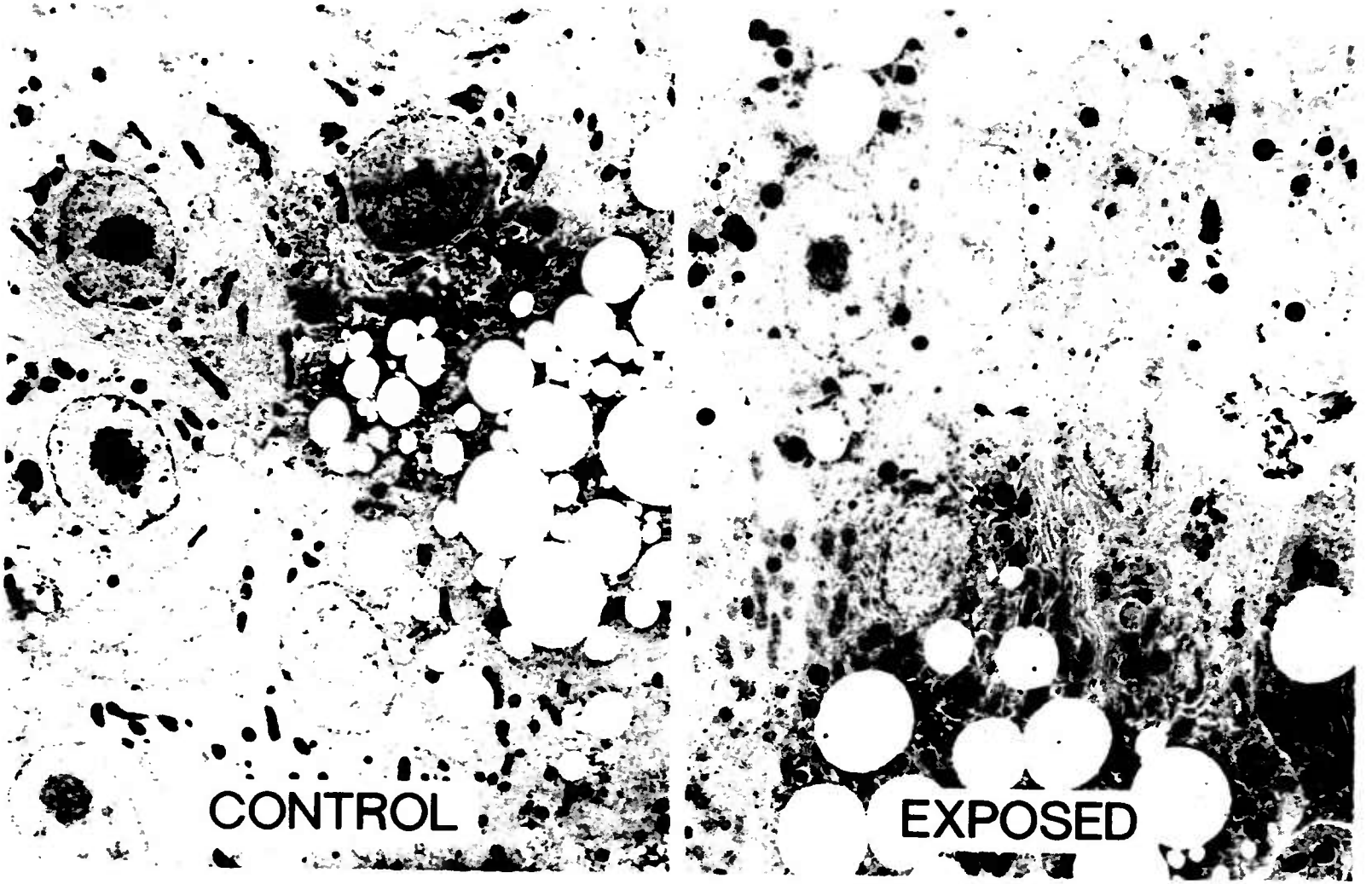

Figure 3. Electron micrographs of liver from control medaka and medaka exposed to DEHP. The peroxisomes are the small, round, darkly stained organelles. There are only 5 to 7 peroxisomes visible in the control sample, while there are 30 to 40 in the exposed sample.

Table 10. Peroxisome Proliferation Results in Livers from Control Medaka and Medaka Exposed to DEHP*

Sample #	Control		Exposed	
	LM	EM	LM	EM
1	++	++	++	++
2	+	+	+++++	++++
3	–	NA	++++	NA
4	+++	NA	+-	NA
5	++	NA	+++	NA

*All livers were examined by light microscopy (LM), while only two from each group were examined by electron microscopy (EM) to corroborate the light microscope observations. The number of pluses indicates relative abundance of peroxisome. One fish had no demonstrable peroxisomes (-) and six of the samples were not examined by EM (NA).

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ANALYSIS OF THE FIVE STEP SECTION METHOD FOR HISTOLOGY OF THE JAPANESE MEDAKA (*Oryzias latipes*)

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INTRODUCTION

The National Toxicology Program has established standards for conducting and evaluating carcinogenicity studies in rodents (Huff et al., 1988). Small aquarium fish species have recently been used in similar types of tests for carcinogenic effects (Hawkins et al., 1985; Prince Masahito et al., 1988; Pliss and Khudoley, 1975), and an interest has developed in adapting to fish some of the standards that have already been used successfully in rodents. Of particular concern are the standards for histopathologic evaluation of tissues. In any toxicologic study, it is important to minimize any type of bias among treatment groups from the time the animal is necropsied until the tissues are evaluated by a pathologist (Haseman et al., 1989). In rodent studies, great care is taken to be sure that a standard set of organs is collected at necropsy and that the tissues are trimmed for sectioning in the same manner for each animal. Bias could be created, e.g., by trimming two sections of liver for some animals and three sections for others or by trimming the spleen longitudinally for some animals and transversely for others.

This report is an evaluation of the longitudinal step section method for sampling the tissues of the Japanese medaka (*Oryzias latipes*). The method is examined, first of all, for tissue accountability; that is, to determine if a standard set of protocol-required tissues could be identified in all specimens of fish sectioned by this method. Secondly, the method is evaluated to check for consistency in the amount of tissue present from fish to fish.

MATERIALS AND METHODS

Tissue Accountability

Japanese medaka from the U.S. Army's Studies G and N were fixed in Bouin's fixative, processed whole through a graded series of ethanol and toluene, and infiltrated with and embedded in paraffin. The fish were sectioned longitudinally with a thickness of 4 to 5 μm such that five step sections were taken through the fish: two right paramedian sections, one

midsagittal section, and two left were mounted on slides and stained with hematoxylin and eosin. There were 459 fish in Study G (245 from the 3-month interim sacrifice and 214 from the 6-month final sacrifice) and 200 fish from the interim sacrifice of Study N. The following tissues were evaluated and accounted for in each fish: bone (vertebra), brain, chromaffin tissue, corpuscle of Stannius, esophagus, eye, gallbladder, gill, heart, hematopoietic tissue, interrenal tissue, intestine, kidney, liver, nares, ovary, pancreas, peripheral nerve, pineal organ, pituitary gland, pseudobranch, skeletal muscle, skin, spinal cord, spleen, stato-acoustic organ, swim bladder, testis, thymus, thyroid tissue, and urinary bladder.

A group of 30 Japanese medaka that were retired breeders 1 to 2 years of age were sacrificed, divided into groups of 10 fish each, fixed and processed as described above, and sectioned longitudinally by three different methods. Ten fish were sectioned according to the five step section method described above. Ten fish had seven step sections taken: three right paramedian sections, one midsagittal section, and three left paramedian sections. Ten fish had nine step sections taken: four right paramedian sections, one midsagittal section, and four left paramedian sections. In all three groups, duplicate sections from each level were mounted on slides and stained with hematoxylin and eosin. The fish from each group were evaluated for the presence of the tissues previously listed.

Morphometry

The areas of brain, heart, kidney, and liver present in histologic sections of Japanese medaka were measured in control fish from the U.S. Army's Study N: 25 fish from the 3-month interim sacrifice, 15 fish from the 6-month sacrifice of the chronic phase of the study, and 11 fish from the 6-month sacrifice of the recovery phase of the study.

The morphometry system that was used consisted of a Leitz projection microscope and a Summa Sketch Plus digitizing pad attached to a Sperry PCIT computer. The computer software used was Sigma Scan version 3.9. The measuring system was calibrated before each session in which organs were measured to assure accuracy. The organs for each fish were measured by tracing around the outer perimeter of the organ in any of each of the five histologic step sections in which it appeared. The posterior limit of the kidney that was measured was at the level of the anterior limit of the swim bladder. In sections in which a large vein divided the renal tissue, the lumen of the vein was not included in the kidney measurement. Both the atrium and ventricle, when present, were measured as heart.

Means and standard deviations were calculated for the length and weight of the fish in each control group and for the areas of the brain, heart, kidney, and liver measured for the

fish in each control group. Linear regression was calculated on length of the fish versus area of each organ and weight of the fish versus area of each organ in fish from the three control groups.

The areas of brain, heart, kidney, and liver were measured in the histologic sections of retired breeder Japanese medaka sectioned by the five, seven, and nine step section methods described earlier under Tissue Accountability. Five fish from each of the three groups were selected for organ measurements. The measurements were made with the same morphometry system that was used with the control fish described above.

The means and standard deviations for the organ measurements for each group were calculated. An analysis of variance (Gad and Weil, 1986) was calculated on the organ measurement values for each step section group for each organ. Dunnett's *t*-test (Gad and Weil, 1986) was used to compare organ area increases between groups.

RESULTS

Tissue Accountability

In the Japanese medaka from the U.S. Army's Studies G and N, there were nine organs that were not present in the five step sections in greater than 10% of the fish. These organs were chromaffin tissue, corpuscle of Stannius, gallbladder, interrenal tissue, pineal organ, pituitary gland, spleen, thymus, and urinary bladder (Table 1). Bone (vertebra), heart, nares, spinal cord, and swim bladder were organs that were not present in the sections in less than 10% of the fish.

In fish sacrificed at 3 months into the study, the most poorly represented organs were corpuscle of Stannius, pituitary gland, pineal organ, and spleen. In fish sacrificed at 6 months into the study, the pineal gland and the pituitary gland continued to be poorly represented. The urinary bladder and the thymus were seen less frequently in the older fish, while there was improvement in the accountability of chromaffin tissue, corpuscle of Stannius, gallbladder, interrenal tissue, and spleen.

Among the retired breeders that were 1 to 2 years old, the identical nine organs described above were not present in 10% or more of the fish when five step sections were cut for each fish (Table 2). When the number of step sections cut was increased to seven and nine, there was improvement in the accountability of most of the organs except for the pineal organ and pituitary gland. The accountability of these two organs actually decreased as the number of sections increased.

Table 1. U.S. Army Studies G and N: Tissue Accountability

	Percent of Fish in Which Tissues Are Absent		
	Study G 3-Month Sacrifice	Study N 3-Month Sacrifice	Study G 6-Month Sacrifice
Chromaffin Tissue	27	23	14
Corpuscle of Stannius	55	46	12
Gallbladder	22	20	12
Interrenal Tissue	25	23	14
Pineal Organ	56	24	49
Pituitary Gland	50	47	59
Spleen	50	45	30
Thymus	12	36	42
Urinary Bladder	33	18	60
	(n = 245)	(n = 200)	(n = 214)

Table 2. Tissue Accountability of Retired Japanese Medaka Breeders

	Percent of Fish in Which Tissues Are Absent		
	5 Steps	7 Steps	9 Steps
Chromaffin Tissue	20	0	0
Corpuscle of Stannius	30	20	10
Gallbladder	10	0	0
Interrenal Tissue	20	0	0
Pineal Organ	30	30	40
Pituitary Gland	30	50	70
Spleen	70	40	0
Thymus	70	50	30
Urinary Bladder	30	0	10
	(n = 10)	(n = 10)	(n = 10)

Consistency in Amount of Tissue Sampled

Among the control Japanese medaka from the U.S. Army's Studies G and N in which five step sections were cut for each fish, the organ with the greatest amount of tissue present in the sections was the liver. Next most abundant was the brain, and the kidney and heart were third and fourth in abundance, respectively (Tables 3, 4, and 5). For all organs in all groups, there was a wide range in the amount of each tissue seen among individual fish. For example, the amount of liver observed in control fish that were sacrificed at 3 months of age varied from 2.33 sq. mm to 12.27 sq. mm. Linear regression analysis for weight of the fish versus amount of each tissue observed and length of the fish versus amount of each tissue observed indicated that there was no consistent relationship of length or weight to the amount of brain, heart, kidney, or liver observed in the fish among the various control groups (see Tables 6 and 7).

Among the retired breeder Japanese medaka that were sectioned in either five, seven, or nine step sections, the amount of tissue observed usually increased as the number of sections increased (Table 8). There was a wide range in the amount of each tissue seen among individual fish. This range was especially wide in the fish with nine step sections (Figure 1). An analysis of variance calculated for the areas of brain, heart, kidney, and liver in each step section group indicated a significant increase in area of brain and liver as the number of step sections increased. For brain, the difference in area was significant between five step sections and nine step sections. For liver, the area was increased significantly between the five step section group and the seven step section group, between groups five and seven, and between groups seven and nine.

DISCUSSION

In the guidelines for tissue accountability in rodent studies conducted by the National Toxicology Program, most tissues are expected to be present for evaluation 94 to 100% of the time in rats. Exceptions are the parathyroid gland and the thymus (in 2-year studies) for which an accountability of 80 to 100% is acceptable. By these standards, tissue accountability in the Japanese medaka sectioned longitudinally by the five step section method would be unacceptable. Nine organs were not represented variously in 12 to 59% of the fish in the U.S. Army's Studies G and N.

The pituitary gland, pineal organ, corpuscle of Stannius, and urinary bladder are located on the midline of the Japanese medaka. Because these tissues are among the most poorly represented tissues, there appears to be a problem with selecting a truly midline section at the time of microtoming. This may be related to whether or not the fish is embedded so that the

Table 3. U.S. Army Study N: Control Fish (3-Month Interim Sacrifice)

(n = 25)

	Mean	Standard Deviation	Range Low	Range High
Length (mm)	21.24	1.97	17	24
Weight (mg)	152.76	44.42	73	251
Brain (sq. mm)	5.24	1.19	3.02	7.12
Heart (sq. mm)	0.91	0.38	0.22	2.02
Kidney (sq. mm)	1.37	0.61	0.61	2.97
Liver (sq. mm)	6.67	2.43	2.33	12.27

Table 4. U.S. Army Study N: Control Fish (6-Month Chronic Sacrifice)

(n = 15)

	Mean	Standard Deviation	Range Low	Range High
Length (mm)	25.73	1.81	22	29
Weight (mg)	286.20	63.50	193	439
Brain (sq. mm)	6.37	1.52	4.51	9.75
Heart (sq. mm)	1.80	0.75	0.68	3.57
Kidney (sq. mm)	2.88	1.71	1.11	6.11
Liver (sq. mm)	10.46	3.26	4.30	15.55

Table 5. U.S. Army Study N: Control Fish (6-Month Recovery Sacrifice)

(n = 11)

	Mean	Standard Deviation	Range Low	Range High
Length (mm)	29.00	1.60	26	31
Weight (mg)	408.10	84.82	270	543
Brain (sq. mm)	8.40	1.62	6.26	11.77
Heart (sq. mm)	2.17	0.65	1.08	3.09
Kidney (sq. mm)	2.65	1.58	1.25	7.14
Liver (sq. mm)	16.03	5.28	7.39	23.28

Table 6. U.S. Army Study N: Linear Regression

(r values)

	Length versus area of			
	Brain	Heart	Kidney	Liver
Control Fish (Interim, n = 25)	.1507	.2113	.5678	.7542
Control Fish (Chronic, n = 15)	.5766	.5079	.5944	.0958
Control Fish (Recovery, n = 11)	.1500	.5338	.3845	.0125

Table 7. U.S. Army Study N: Linear Regression

(r values)

	Weight versus area of			
	Brain	Heart	Kidney	Liver
Control Fish (Interim, n = 25)	.1643	.2829	.5667	.7797
Control Fish (Chronic, n = 15)	.5735	.6910	.6519	.1022
Control Fish (Recovery, n = 11)	.1663	.5892	.3444	.1960

Table 8. Japanese Medaka Retired Breeders: Means and Standard Deviations for Organ Areas in the Five, Seven, and Nine Step Section Groups

(n = 5 in each group)

	Brain	Heart	Kidney	Liver
Five Steps				
Mean Area	6.61	2.21	3.12	11.03
Standard Deviation	1.45	0.79	1.70	2.84
Seven Steps				
Mean Area	8.55	2.04	3.20	16.81
Standard Deviation	0.89	0.59	1.62	4.68
Nine Steps				
Mean Area	10.71	3.24	6.18	23.72
Standard Deviation	2.07	1.05	3.87	4.93

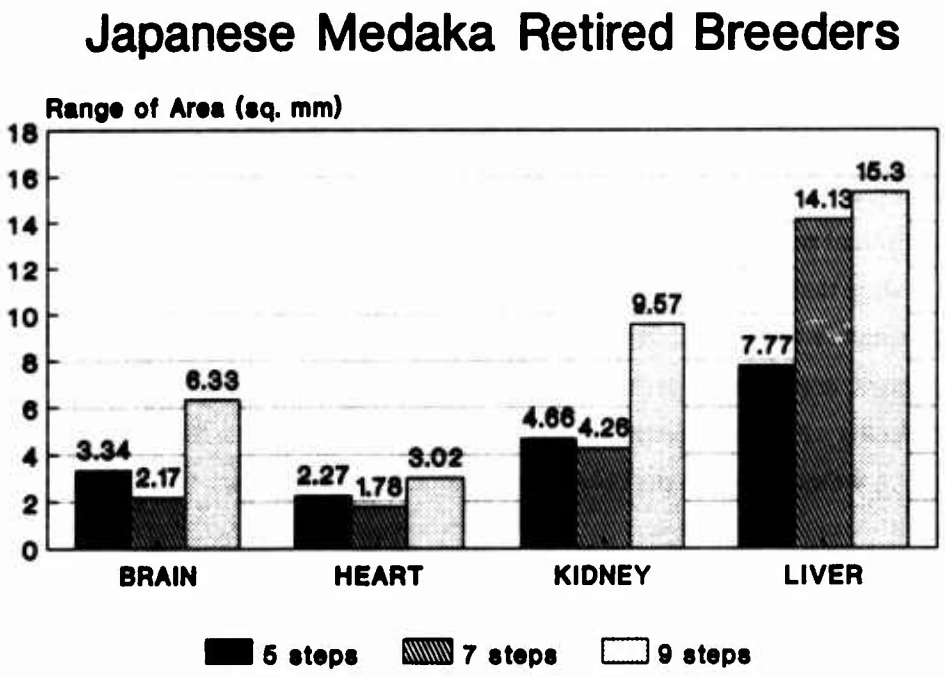

Figure 1. Comparison of five, seven, and nine step sections of the brain, heart, kidney, and liver of Japanese medaka retired breeders.

longitudinal axis is precisely parallel to the surface of the block. The organs that are poorly represented in the sections are also relatively small with the possible exceptions of the spleen and gallbladder, which may be variable in size.

In the study in which the number of step sections was increased from five to nine, there was improvement in tissue accountability to 90 to 100% except for the pituitary gland, pineal organ, and thymus, which continued to have poor accountability. The question that must be answered is whether the improvement in tissue accountability with nine longitudinal step sections is worth the increased cost for slide production and histologic evaluation. It is possible that well-defined cross sections of the Japanese medaka would result in improved tissue accountability.

One additional matter of tissue accountability that has not been addressed in the studies described above is that of accounting for two of paired organs, such as the eye, pseudobranch, thymus, gill (actually four gill arches on each side of the head), kidney, ovary, testis, nares, and stato-acoustic organ. Both organs of a pair are routinely sampled and evaluated in the National Toxicology Program rodent bioassays. It would seem that cross sections of the fish as suggested above would increase the chances that both organs of a pair would be present for evaluation.

Brain, heart, kidney, and liver were chosen for analysis of consistency in amount of tissue sampled because these organs are relatively large, fairly constant in size, and are present 100% of the time in the five longitudinal step sections. These organs were present in one to all five of the step sections examined in the control fish from the U.S. Army's Study N. The heart was occasionally found in only one of the sections. The liver was usually present in all five sections. Parts of the brain were present in three to five of the sections, and the kidney was present in two to five of the sections. The larger the organ, the greater was the range of areas present among the fish for that organ. There was little, if any, correlation between the size of the fish (length and weight) and the area of the organs present in the sections. In the study of five, seven, and nine step sections, increasing the number of step sections to nine not only increased the amount of brain, heart, kidney, and liver observed, but also increased the range of areas observed for these organs. These results indicate that the longitudinal step section sampling method for Japanese medaka tissues yields a high degree of inconsistency in the amount of tissue sampled from one fish to another. It is not known if this degree of inconsistency in tissue sampling in fish would bias the results of a carcinogenesis bioassay. It would seem that this area of concern should be investigated further.

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DEVELOPMENT OF IMMUNOTOXICOLOGICAL ASSAYS FOR THE JAPANESE MEDAKA: GENERATION OF CYTOTOXIC OXYGEN RADICALS BY PHAGOCYTIC BLOOD CELLS USING A BIVALVE MODEL SYSTEM

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BACKGROUND

Multicellular organisms, both invertebrate and vertebrate, cold-blooded or homeothermic, all use various classes of phagocytic cells to protect against microbial infection, parasitism, and other potential pathogenic agents that gain access to the blood. These phagocytes kill foreign microorganisms either intracellularly or extracellularly by several mechanisms mediated by cytotoxic molecules. The chemicals involved in these cidal reactions include a number of hydrolases such as lysozymes, which are derived from lysosomes, and several biologically active oxygen metabolites.

Because of the central importance of cytotoxic oxygen radicals to host defense mechanisms and because of the highly sensitive methods available for their quantitation, these parameters are being examined for utility in immunotoxicity protocols using the medaka. In order to develop the capability to perform the assays at the Chesapeake Biological Laboratory, the researchers have chosen to use blood cells from the oyster, *Crassostrea virginica*, as a model system.

This is appropriate for preliminary studies for two reasons. First, an abundant supply of *C. virginica* hemocytes is readily available, whereas the supply of circulating blood cells from the medaka is limited by its small size. Second, macrophages (the specific cells under study) have been shown to be remarkably similar in all species studied; a number of morphological and physiological similarities between bivalve hemocytes and vertebrate macrophages have already been demonstrated (Anderson et al., 1981; Anderson and Döös, 1983; Anderson, 1987; Anderson, 1988). Therefore, there is reason to believe that the methodology developed with oyster cells can be extrapolated to fish macrophages. The involvement of cytotoxic oxygen species in cellular defense mechanisms appears to be a highly conserved trait at all phyletic levels.

When phagocytes are stimulated by particle ingestion or appropriate membrane phenomena, they manufacture a number of powerful oxidizing agents. This event is

accompanied by increased oxygen consumption; the metabolic events associated with this are called the respiratory burst. The ultimate source of the microbicidal oxidants is the superoxide radical (O_2^-), which is produced in relatively large quantities after stimulation (Babior et al., 1988). The production of O_2^- is accomplished by one electron reduction of O_2 at the expense of NADPH:

$$2O_2 + NADPH \xrightarrow[\text{burst oxidase}]{\text{respiratory}} 2O_2^- + NADP^+ + H^+.$$

The dismutation of O_2^- to H_2O_2 and O_2 can occur spontaneously or can be facilitated by specific enzymes:

$$2O_2^- + 2H^+ \xrightarrow[\text{dismutase}]{\text{superoxide}} H_2O_2 + O_2 .$$

Hydrogen peroxide is highly toxic and, furthermore, in the presence of chloride ions and myeloperoxidase, forms the most potent antimicrobial system yet identified in phagocytes (Klebanoff, 1968). However, there is also considerable evidence that O_2^- alone can exert numerous direct toxic effects on bacteria (Fridovich, 1988). Other oxidants associated with the respiratory burst have also been implicated in cytotoxicity, such as singlet oxygen and the hydroxyl radical (Britigan et al., 1986).

Methodology already developed for the study of cytotoxic oxygen intermediates in mammals has been applied to the quantitation of these responses in shellfish and finfish. The methods are precise, and the results are highly reproducible. The assays are quite sensitive, allowing detection of oxygen radicals at nanomolar concentrations and the use of relatively small numbers of phagocytes (2×10^6). Therefore, these assays will be of use in the study of medaka immune responses, when cell numbers/animal are limiting.

QUANTITATIVE NITROBLUE TETRAZOLIUM ASSAY

The reduction of nitroblue tetrazolium (NBT) is recognized as a measure of the oxidative events associated with cell-mediated killing and has been used clinically to identify defects in blood cell defense capabilities (Park et al., 1968). NBT is a pale yellow dye that is reduced by O_2^- to a dark blue-black, water-insoluble product called formazan. Normal phagocytes show markedly enhanced formazan production during phagocytosis or when their membranes are stimulated by an appropriate soluble chemical, such as phorbol myristate acetate (PMA). NBT

reduction in blood cells was classically evaluated under the microscope by observing the characteristic deposition of formazan reaction products around ingested particles. To measure NBT reduction more precisely, the slide method was abandoned in favor of the quantitative spectrophotometric analysis of pyridine extracts of hemocyte preparations; this method was modified from that of Baehner and Nathan (1968).

The protocol for quantitative NBT reduction is summarized in Figure 1. Briefly, several tubes containing ~2×10^6 hemocytes are suspended in 0.025% NBT. The tubes are incubated for 60 minutes, with gentle mixing, either in the presence of 10 mg/ml heat-killed, washed yeast cells (for phagocytic stimulation) or in the absence of yeast (to measure resting NBT reduction levels). The reaction is stopped by the addition of 1.0 N HCl, and the formazan and cell debris are removed from suspension by centrifugation. The pellet is extracted with pyridine (100°C for 30 minutes), and the extracts are clarified by further centrifugation. The supernatants are read in a spectrophotometer at 515 nm against a pyridine blank; results are expressed as Δ $OD_{515\ nm}$ /2×10^6 cells/60 minutes.

Representative results from the quantitative NBT assay are summarized in Table 1. The resting and phagocytically stimulated levels of NBT reduction show some degree of natural variation; however, the average OD_{515} increment upon stimulation averages about 70%. This stimulated increase was reduced to about 30% by the presence of 600 U of superoxide dismutase (SOD) in the culture medium. It was encouraging that much of the phagocytically induced increment in NBT reduction was sensitive to SOD, indicating the required level of O_2^- dependency for this response.

Table 1. Representative Results from the Quantitative NBT Assay

Quantitative NBT Reduction
Resting level range: 0.033-0.095 $OD_{515}/2\times10^6$ hemocytes
Phagocytizing level range: 0.054-0.205 $OD_{515}/2\times10^6$ hemocytes
Average stimulated increment: 68.9±15.7%
Average stimulated increment in the presence of 600 U SOD: 30.2±6.9%

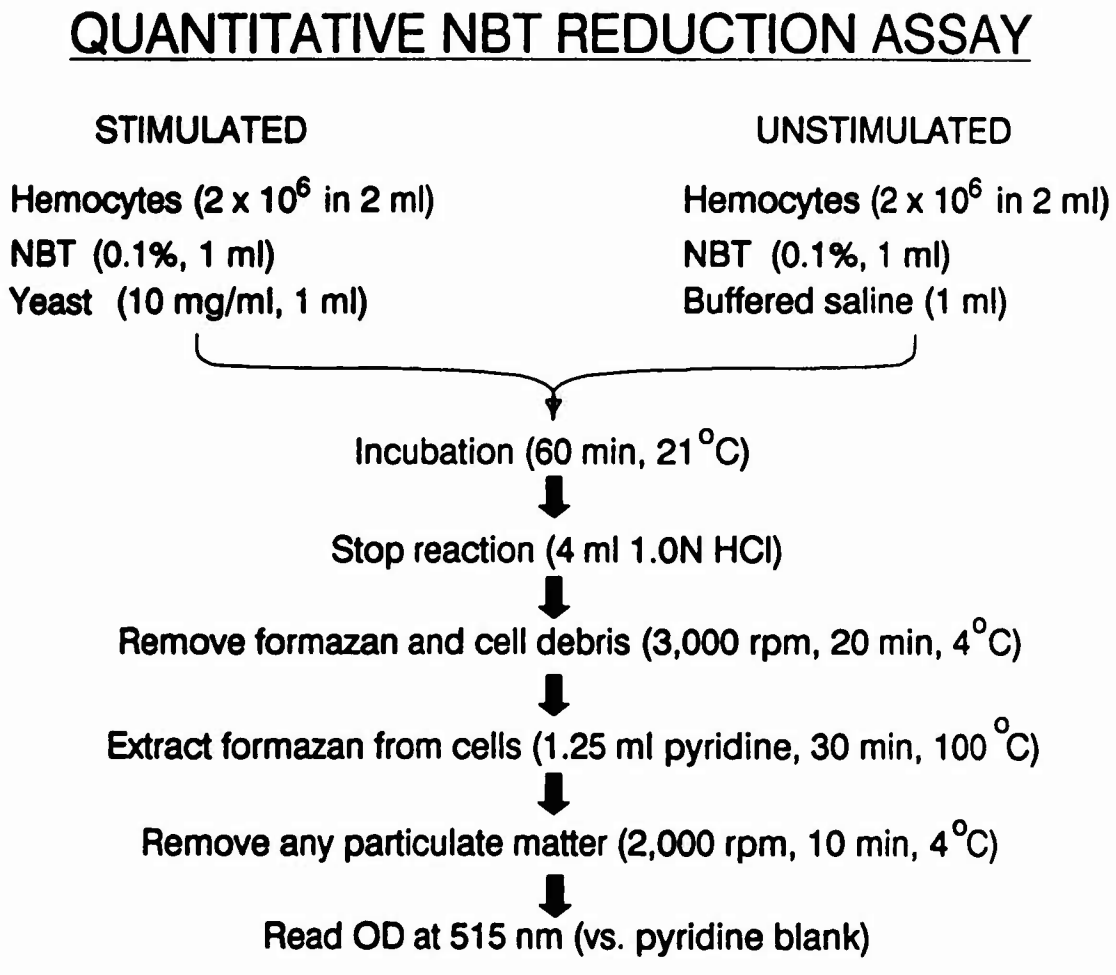

Figure 1. The protocol for quantitative NBT reduction.

The quantitative NBT reduction assay will provide an accurate measure of O_2^- generation by blood cells, an indication of the presence of active or past bacterial infections, and detailed information on specific defects in hemocyte bactericidal capability. Blood cells from uninfected, normal animals that are not phagocytically stimulated show little NBT reduction; however, cells from animals with bacterial infections show considerably increased NBT reduction, even in the absence of experimental stimulation. This may provide an indirect measure of pollutant-induced immunosuppression resulting from the well-documented synergy between chemical stress and decreased resistance to infection. On the other hand, inability to respond to experimental phagocytic stimulation with a typical increase in NBT reduction could also be a direct consequence of immunotoxicity.

H_2O_2 GENERATION ASSAY

A simple method to quantify H_2O_2 release by phagocytic cells *in vitro* is summarized in Figure 2; it follows the protocol of Pick and Keisari (1980). It is based on the H_2O_2-mediated and horseradish peroxidase (HRPO)-dependent oxidation of phenol red to a product with an absorption maximum at 610 nm. The hemocytes (2×10^6) are established as cell monolayers in 3-cm culture dishes, and stimulated cultures are produced by overlaying with 1 μm PMA in phosphate-buffered saline (10 mM PBS, pH 7.0) for 30 minutes at 21°C; unstimulated monolayers are covered with PMA-free phosphate-buffered solution. After this incubation, all monolayers are gently washed and covered with 1 ml of 10 mM phosphate-buffered saline (pH 7.0) containing 5.5 mM dextrose, 8.5 U/ml HRPO, and 0.28 mM phenol red. A phenol-red blank solution is also prepared by incubation in a culture dish in the absence of hemocytes. After 60 minutes incubation at 21°C, the supernatants are withdrawn from each culture dish, any cells are removed by centrifugation (2,000 rpm, 4°C, 5 minutes), the solutions are made alkaline by the addition of 10 μl of 1N NaOH, and the optical density (OD) is read at 610 nm against the phenol-red solution blank similarly treated with NaOH. The absorbancy readings are converted to H_2O_2 concentrations via a standard curve (Figure 3). A linear relationship exists between $OD_{610\ nm}$ and H_2O_2 concentrations in the range of 0.1 to ~30.0 μM, corresponding to concentrations produced by the experimental conditions.

Table 2 shows the effect of various preincubation periods with 1 μM PMA on the subsequent quantification of H_2O_2 production by the phagocytic hemocytes. It must be remembered that because the preincubation medium is removed prior to incubation with the phenol-red solution, the H_2O_2 generated during preincubation (stimulation) cannot be

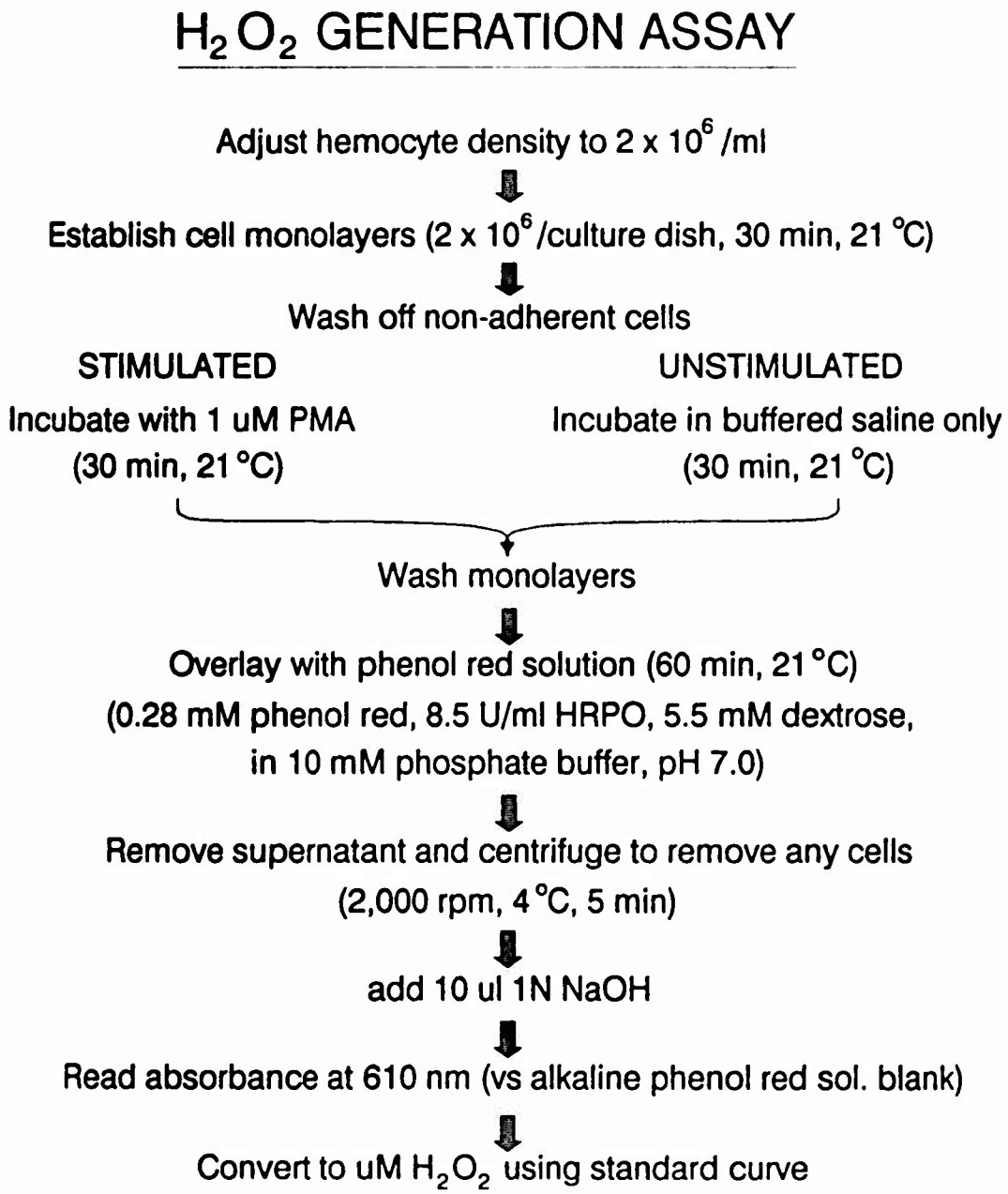

Figure 2. Method for quantifying H_2O_2 release by phagocytic cells *in vitro*.

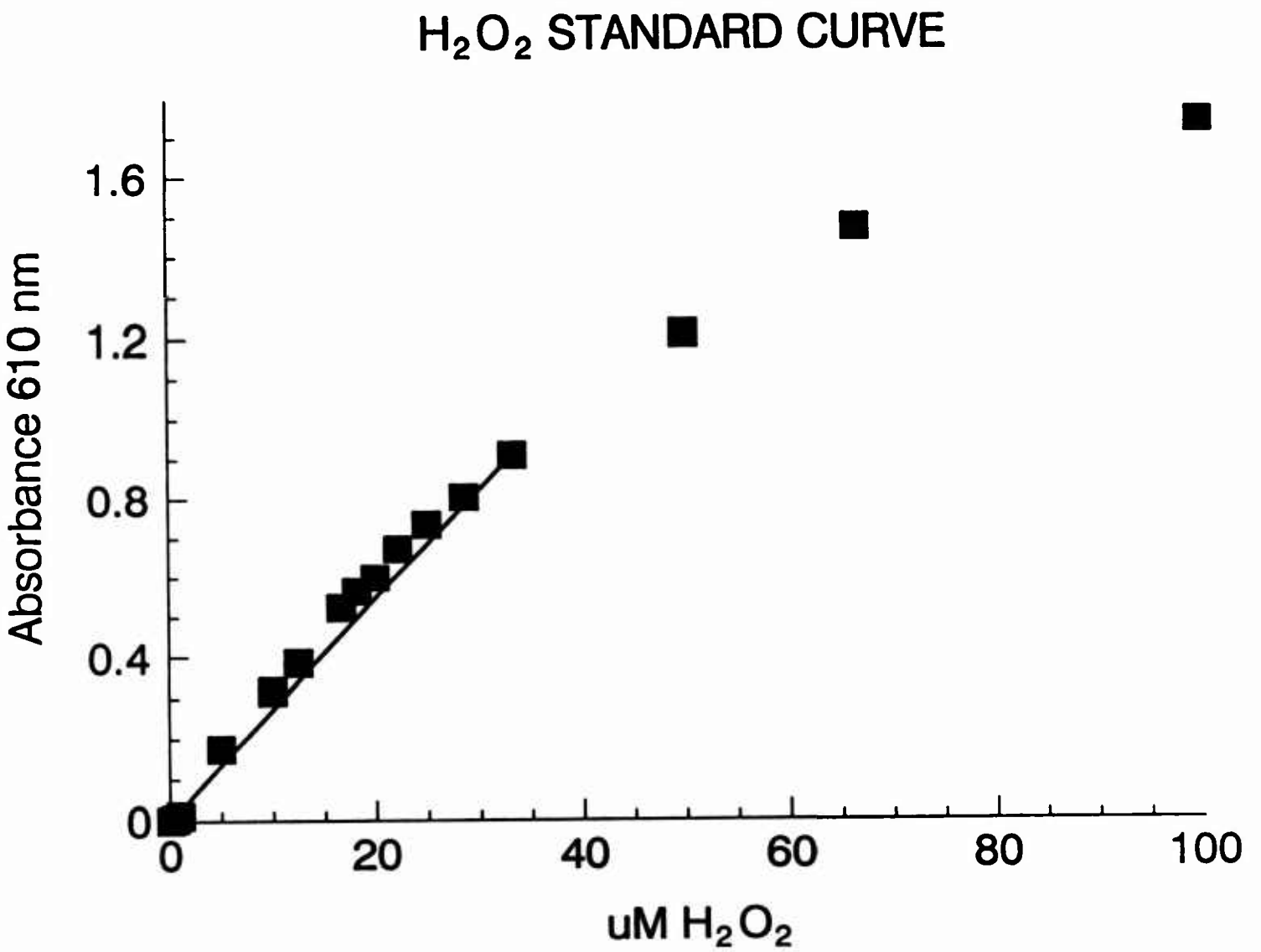

Figure 3. Standard curve for converting absorbancy readings to H_2O_2 concentrations.

quantified. Therefore, the researchers wished to define an adequate preincubation time required to stimulate activity and permit recovery of a representative H_2O_2 sample. The data indicate that H_2O_2 production is not continuous after PMA stimulation. The amount produced after 30 minutes was only slightly less than after 10 minutes, but after 60 minutes production had markedly decreased. It is interesting to see that unstimulated cells also stopped releasing H_2O_2 with time, with essentially no production after 60 minutes in phosphate-buffered saline. The explanation for the gradually decreasing release of H_2O_2 by the non-PMA-stimulated cells is not known; perhaps the cells are minimally stimulated during the adhesion process when the monolayers are forming.

Table 2. Effect of Preincubation Period Duration on H_2O_2 Production

	$\bar{x}$ OD_{610} Stimulated*	$\bar{x}$ OD_{610} Resting	Δ OD_{610}
10 minutes	0.210	0.059	0.151
30 minutes	0.162	0.029	0.133
60 minutes	0.044	0.000	0.044

*1 μM PMA.

The concentration of PMA to which the cells were exposed during preincubation was varied to determine the most effective stimulatory dose (Table 3). The response was dose dependent from 0.1 μM to 1.0 μM, but further increases in PMA concentration were not effective. The researchers have used 1.0 μM PMA as the standard concentration for H_2O_2 stimulation in all additional assays. There had been reports that H_2O_2 production *in vitro* was dependent on cell density in the culture vessels, with inhibition being seen at high densities. A similar effect with *C. virginica* hemocytes could not be demonstrated (Table 4). Total H_2O_2 production, both stimulated and unstimulated, continued to rise with increasing cell densities up to 4×10^6, which is about the total binding capacity of the culture dishes.

SUPEROXIDE GENERATION ASSAY

The superoxide anion is measured by the reduction of ferricytochrome c by the general method of Pick and Mizel (1981); the procedure is summarized in Figure 4. Hemocyte

Table 3. Effect of PMA Concentration During 30-Minute Preincubation on H_2O_2 Production

μM PMA	$\bar{x}$ OD_{610}
0 (unstimulated)	0.063
0.1	0.090
0.5	0.145
1.0	0.161
10.0	0.144
20.0	0.130
50.0	0.098
100.0	0.067

Table 4. Effect of Cell Density on H_2O_2 Generation

	Unstimulated Δ OD_{610}	Stimulated* Δ OD_{610}
5×10^5	0.000	0.005
1×10^6	0.000	0.035
2×10^6	0.022	0.136
4×10^6	0.042	0.238

*1 μM PMA.

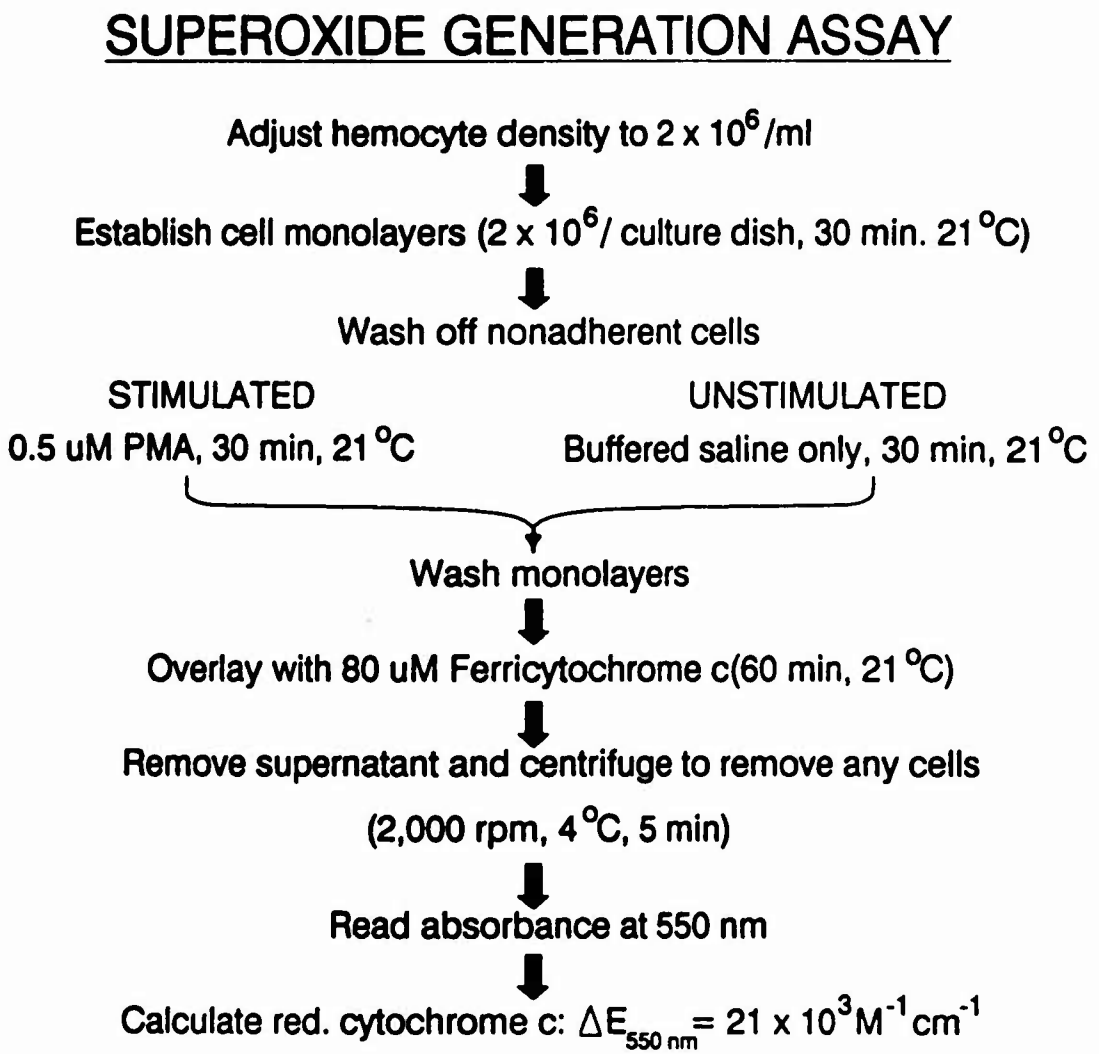

Figure 4. Procedure for superoxide generation assay.

monolayers (2×10^6 cells/dish) are stimulated with 0.5 μM PMA for 30 minutes at 21°C; unstimulated phagocytes are incubated under comparable conditions without PMA. The monolayers are then washed and covered with 80 μM ferricytochrome c (Sigma, Type III) and incubated for 60 minutes at 21°C. The supernatants are removed and cleared of cells and particulates by centrifugation (2,000 rpm, 4°C, 5 minutes). The OD is measured at 550 nm against blanks of cytochrome c incubated in the absence of hemocytes. The O_2^- generated is calculated from the extinction coefficient for reduced cytochrome c minus oxidized cytochrome c, as read at 550 nm, by the formula: $\Delta E_{550\,nm} = 21\times10^3\ M^{-1}\ cm^{-1}$. A procedure similar to that described for optimization of the H_2O_2 assay was followed to establish optimal conditions for the O_2^- assay. O_2^- production was maximally stimulated at 0.5 μM PMA using a 30-minute preincubation period; PMA concentrations >10 μM were inhibitory, suggesting cytotoxicity at this level. As was the case for H_2O_2 production, O_2^- release is stimulated within 5 minutes of PMA exposure. However, unlike the experience with H_2O_2, superoxide release by both unstimulated and stimulated phagocytes seems to persist at a relatively steady level over a longer period of time (Table 5). There was no inhibition of O_2^- production at high cell densities, up to 4×10^6, similar to the results with H_2O_2.

Table 5. Effect of Preincubation Period Duration on Superoxide Production

	$\bar{x}$ OD_{550} Stimulated*	$\bar{x}$ OD_{550} Resting	Δ OD
5 minutes	0.185	0.154	0.031
10 minutes	0.177	0.130	0.047
20 minutes	0.167	0.125	0.042
30 minutes	0.148	0.107	0.041
45 minutes	0.134	0.111	0.023

*0.5 μM PMA.

CONCLUSIONS

The ability of this laboratory to quantify the cytotoxic, protective capacity of phagocytic blood cells has been expanded by adding several assays for biologically active oxygen radicals. It is also planned to add a cellular chemiluminescence assay that quantifies the generation of photons by cells during phagocytic activation. This assay is very sensitive and is accepted as an accurate measure of cellular killing capacity. Current capabilities for measuring intracellular and extracellular toxic oxygen radicals associated with resting (unstimulated) and stimulated blood phagocytes are summarized in Figure 5. These assays will be used to study the potential immunosuppressive effects of environmental chemicals. In this way, insight will be gained regarding mechanisms of pollutant-induced immune dysfunction, and unique immunotoxicological assays for the evaluation of sublethal effects of xenobiotics may also be developed using the medaka model system.

Figure 5. Capabilities for measuring intracellular and extracellular toxic oxygen radicals.

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APPENDIX A

AGENDA FOR 1988 RESEARCH REVIEW MEETING

WORKSHOP ON NON-MAMMALIAN TOXICITY ASSESSMENT RESEARCH REVIEW

U.S. Army Biomedical Research and Development Laboratory
Building 568, Conference Room
Fort Detrick
Frederick, Maryland

23 August 1988

Time	Topic	Presenter/Organization
0800	Welcome	Dr. Thomas Miller, USABRDL
0805	Introduction	Mr. Henry S. Gardner, USABRDL
0810	Research Methods Branch, Introduction	Dr. William van der Schalie, USABRDL
0820	The Use of Fish in On-Site Carcinogenicity Assessment: A Programmatic Overview	Mr. Henry S. Gardner, USABRDL
0845	The Effects of Diethylnitrosamine on Hepatic Ornithine Decarboxylase Activity in Small Fish	Ms. Linda Brennan, USABRDL
0915	BREAK	
0930	New Models for Oncogene Isolation in the Study of Carcinogenesis	Dr. Rebecca J. Van Beneden, NCI Johns Hopkins University
1000	Ultrastructural Characterization of Selected Chemically Induced Lesions in Medaka	Dr. William N. Norton S.E. Louisiana University
1030	Pathology of DEN Induced Lesions in the Medaka	Dr. Tracie E. Bunton Johns Hopkins University School of Medicine
1100	LUNCH	
1230	New Models for Epigenetic Carcinogens	Dr. Edward Calabrese University of Massachusetts
1300	Development of New Carcinogenicity Bioassays	Dr. William Hawkins Gulf Coast Research Laboratory
1330	U.S. Environmental Protection Agency Collaborative Research in New Toxicity Methods	Dr. Rodney Johnson Duluth EPA Environmental Research Laboratory
1400	BREAK	

Time	Topic	Presenter/Organization
1420	National Cancer Institute Research in New Carcinogenicity Models	Dr. David Longfellow National Cancer Institute
1440	Tumor and Neoplasia Mechanism Studies	Dr. Jerry D. Hendricks Oregon State University
1510	Future Research Plans	Mr. Henry S. Gardner, USABRDL
1530	Discussion	All Participants
1630	Conclusion	Mr. Henry S. Gardner, USABRDL
1800	No-Host Dinner	All Participants

APPENDIX B

LIST OF INVITEES FOR 1988 RESEARCH REVIEW MEETING

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APPENDIX C

AGENDA FOR 1989 RESEARCH REVIEW MEETING

SECOND ANNUAL RESEARCH METHODS BRANCH WORKSHOP

U.S. Army Biomedical Research and Development Laboratory
Fort Detrick
Frederick, Maryland

8-9 August 1989

8 August

Time	Topic	Presenter/Organization
0800	Welcome	Col. Steve Hembree Commander, USABRDL
0805	Introduction	Dr. Irv Baumel Chief, Health Effects Research Division
0810	Research Methods Branch Overview	Dr. William van der Schalie Chief, Research Methods Branch USABRDL
0830	Current Issues in Carcinogenicity Testing	Dr. Robert Squire Division of Comparative Medicine Johns Hopkins University
0915	USABRDL Toxicity Assessment Methods Development and Application	Mr. Henry S. Gardner Research Methods Branch USABRDL
1000	BREAK	
1015	Pathology of DEN-Induced Lesions in the Medaka	Dr. Tracie Bunton Johns Hopkins University
1045	Development of In Vitro Techniques with Medaka	Dr. Sandra Baksi Environmental Research Laboratory EPA, Narragansett, RI
1115	LUNCH	
1300	Molecular and Cellular Markers of Toxicity	Dr. John McCarthy Oak Ridge National Laboratory
1340	Assessment of DNA Modifications in Medaka	Dr. Donald Malins Pacific Northwest Research Foundation

Time	Topic	Presenter/Organization
1415	BREAK	
1430	Role of Oncogenes in Chemical Carcinogenesis	Dr. Rebecca Van Beneden Duke University Marine Laboratory
1500	Neoplasia: Tumor and Mechanism Studies	Dr. Jerry Hendricks Oregon State University
1545	DISCUSSION	
1645	BREAK	
1800	NO HOST DINNER	

9 August

0800	INTRODUCTION	Mr. Henry S. Gardner USABRDL
0810	Dose-Response Relationships with Low Concentrations of DEN	Dr. William Walker Gulf Coast Research Laboratory
0830	New Methods for Epigenetic Carcinogens	Dr. Edward Calabrese University of Massachusetts
0910	Development of Carcinogenesis Bioassay Models	Dr. William Hawkins Gulf Coast Research Laboratory
0945	BREAK	
1000	Medaka Carcinogen Assay Validation	Dr. Rodney Johnson Environmental Research Laboratory EPA, Duluth, MN
1040	Histopathology Protocol Issues	Dr. Marilyn Wolfe Experimental Pathology Laboratories
1100	Immunotoxicology Assessment Using Small Fish	Dr. Robert Anderson University of Maryland
1120	DISCUSSION	
1200	CLOSE	

APPENDIX D

LIST OF INVITEES FOR 1989 RESEARCH REVIEW MEETING

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